



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

de la MONTE *et al.*

Appl. No. 09/964,412

Filing date: September 28, 2001

For: **Transgenic Animals and Cell  
Lines for Screening Drugs  
Effective for the Treatment or  
Prevention of Alzheimer's Disease**

Confirmation No.: 2051

Art Unit: 1635

Examiner: McGarry, S.

Atty. Docket: 0609.4370004/RWE/FRC

**Brief on Appeal Under 37 C.F.R. § 41.37**

*Mail Stop Appeal Brief - Patents*

Commissioner for Patents  
PO Box 1450  
Alexandria, VA 22313-1450

Sir:

A Notice of Appeal from the final rejection of claims 35-42 was filed on December 1, 2004. Appellants hereby file this Appeal Brief, together with the required brief filing fee.

It is not believed that extensions of time are required beyond those that may otherwise be provided for in documents accompanying this paper. However, if additional extensions of time are necessary to prevent abandonment of this application, then such extensions of time are hereby petitioned under 37 C.F.R. § 1.136(a), and any fees required therefor (including fees for net addition of claims) are hereby authorized to be charged to our Deposit Account No. 19-0036.

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***I. Real Parties In Interest***

The real parties in interest in this appeal are The General Hospital Corporation and Nymox Corporation.

***II. Related Appeals and Interferences***

Appeals to the Board of Patent Appeals and Interferences have been filed in the following related patent applications:

- 09/964,678      Appeal Brief filed: October 8, 2003  
Reply Brief filed: February 19, 2004  
Request for Oral Hearing filed: February 19, 2004  
Appeal No.: 2004-2135  
Oral Hearing scheduled: April 19, 2005
- 09/380,203      Appeal Brief filed: March 11, 2004  
Revised Appeal Brief filed: April 2, 2004  
Reply Brief filed: November 15, 2004  
Request for Oral Hearing filed: November 15, 2004  
Appeal No.: *to be assigned*
- 09/964,667      Notice of Appeal filed: February 8, 2005

***III. Status of Claims***

Claims 35 and 37-42 are pending in the application.

Claims 1-34, 36 and 43-52 have been canceled.



Claims 35 and 37-42 are rejected.

***IV. Status of Amendments***

Subsequent to the Final Office Action dated June 1, 2004, claims 36 and 43-52 were canceled and claim 35 was amended.

As of the present date no Advisory Action has been received in this application. Applicants' undersigned representative has contacted the Examiner on two separate occasions and has informed the Examiner that no Advisory Action has been received. In a telephone conversation on January 10, 2005, the Examiner informed the undersigned that the after-final amendments set forth in Applicants' response filed August 30, 2004 have been entered but that the rejections under 35 U.S.C. § 112, first paragraph are maintained.

***V. Summary of Claimed Subject Matter***

The present invention relates to the discovery of a protein known as AD7c-NTP which is expressed at high levels in the brains of Alzheimer's disease patients. *See* specification at page 5, lines 3-11, at page 41, lines 18-28, at page 44, lines 2-5, at page 47, lines 7-9, and Figure 2D. AD7c-NTP is encoded by the cDNA of SEQ ID NO:1. *See* specification at page 7, lines 16-17. Overexpression of AD7c-NTP in neuronal cells *in vitro* caused cellular abnormalities characteristic of Alzheimer's disease. *See* specification at page 46, lines 1-26. The present invention therefore relates generally to

methods of treating dementias of the Alzheimer's type of neuronal degeneration by interfering with the expression of AD7c-NTP. In particular, the invention relates to the use of antisense oligonucleotides to interfere with AD7c-NTP expression.

Antisense oligonucleotides are short synthetic DNA or RNA molecules that are complementary to a specific gene or RNA message. *See* specification at page 24, lines 17-18. Binding of antisense oligonucleotides to a target DNA or mRNA can selectively block transcription or translation of the targeted gene, thereby halting the disease process generated by the targeted gene. *See* specification at page 24, lines 19-23.

Claim 35 is the sole independent claim pending in the present application. Claim 35 defines a method for the treatment of dementias of the Alzheimer's type of neuronal degeneration. The method comprises administering to an animal in need thereof an antisense oligonucleotide which is complementary to an NTP mRNA sequence corresponding to nucleotides 150-1139 of SEQ ID NO:1.

Support for claim 35 can be found throughout the specification, for example, at page 7, lines 11-13, at page 24, line 10, through page 27, line 26, in claim 33 as originally filed, and in Figure 1 (depicting SEQ ID NO:1).

#### ***VI. Grounds of Rejection to be Reviewed on Appeal***

Claims 35 and 37-42 stand rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the enablement requirement. *See* Final Office Action dated June 1, 2004 ("Final Office Action"), page 2.

## ***VII. Argument***

### ***A. Legal Standard for Enablement***

The enablement requirement is satisfied if one skilled in the art, after reading the specification, could practice the claimed invention without undue experimentation. *See Chiron Corp. v. Genentech, Inc.*, 363 F.3d 1247, 1253 (Fed. Cir. 2004); *In re Wands*, 858 F.2d 731, 736-37 (Fed. Cir. 1988). A patent disclosure need not enable information within the knowledge of an ordinary skilled artisan. *Chiron*, 363 F.3d at 1254. Thus, "a patentee preferably omits from the disclosure any routine technology that is well known at the time of the application. *Id.*; *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986).

The need for experimentation to practice an invention does not, by itself, indicate lack of enablement; what is required is that the amount of experimentation "must not be unduly extensive." *Chiron*, 363 F.3d at 1253 (quoting *PPG Indus., Inc. v. Guardian Indus., Corp.*, 75 F.3d 1558, 1564 (Fed. Cir. 1996)).

Factors that are considered in determining if the amount of experimentation needed to practice a claimed invention is "undue" include: (1) the breadth of the claims; (2) the nature of the invention; (3) the state of the prior art; (4) the level of one of ordinary skill in the art; (5) the level of predictability in the art; (6) the amount of direction provided by the inventors; (7) the existence of working examples; and (8) the quantity of experimentation needed to make or use the invention based on the content of the disclosure. *See Wands*, 858 F.2d at 737.

In order to establish a *prima facie* case of non-enablement, the Examiner has the initial burden of establishing a reasonable basis to question the enablement provided for the claimed invention. *See In re Wright* 999 F.2d 1557, 1562 (Fed. Cir. 1993). To satisfy this burden, "it is incumbent upon the Patent Office. . . to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *See In re Marzocchi*, 439 F.2d 220, 224, (CCPA 1971) (emphasis in original). The minimal requirement in making an enablement rejection is for the Examiner to give reasons for the uncertainty of the enablement. *See In re Bowen*, 492 F.2d 859, 862-63 (CCPA 1974). This standard is applicable even when there is no evidence in the record of operability without undue experimentation beyond the disclosed embodiments. *See id.*

As explained in detail below, a proper analysis of the relevant "undue experimentation factors" set forth in *Wands* indicates that the practice of the presently claimed methods would *not* have required undue experimentation on the part of one of ordinary skill in the art. In addition, the Examiner has not presented sound arguments or scientific reasoning to explain why it is believed that the practice of the presently claimed methods would have required undue experimentation on the part of one of ordinary skill in the art. Thus, a *prima facie* case of non-enablement has not been established and the rejection cannot be properly maintained.

***B. The Subject Matter of the Present Claims is Fully Enabled***

***1. Breadth of the Claims***

The scope of the present claims is commensurate with the teachings of the specification and the knowledge possessed by persons of ordinary skill in the art.

Independent claim 35 encompasses methods for the treatment of dementias of the Alzheimer's type of neuronal degeneration. The methods comprise administering to an animal in need thereof an antisense oligonucleotide which is complementary to an NTP mRNA sequence corresponding to nucleotides 150-1139 of SEQ ID NO:1. Thus, the claim specifies the animals to which the antisense oligonucleotide is administered (*i.e.*, an animal in need of treatment of dementias of the Alzheimer's type of neuronal degeneration), and also specifies the characteristics of the antisense oligonucleotide that is administered (*i.e.*, that it is complementary to an NTP mRNA sequence corresponding to nucleotides 150-1139 of SEQ ID NO:1).

The present claims are therefore not unduly broad, especially in view of the teachings in the specification (see Section VII.B.6, below) and the level of knowledge and skill and predictability in the art (see Sections VII.B.3-5, below).

***2. Nature of the Invention***

The present invention relates to antisense-based therapies for the treatment of dementias of the Alzheimer's type of neuronal degeneration in animals.

### 3. *State of the Prior Art*

The state of the prior art is what one skilled in the art would have known, at the time the application was filed, about the subject matter to which the claimed invention pertains. M.P.E.P. § 2164.05(a). As discussed below, the state of the art relating to the therapeutic use of antisense molecules, at the time of the effective filing date of the present application, was well established. The advanced state of the art is evidenced by, *inter alia*, several examples showing the successful clinical application of therapeutic antisense molecules.

As of the effective filing date of the present application, it was well known that antisense oligonucleotides can selectively block expression of a target gene by hybridizing to complementary sequences within the target mRNA encoded by the gene. *See* specification at page 24, lines 10-23 and references cited and incorporated by reference therein.

Methods of administering polynucleotides in general had been well established at the time of the effective filing date of the present application, including the use of liposomes, virions, and modified donor cells. *See* specification at page 26, lines 17-24 and at page 32, lines 4-27 and references cited and incorporated by reference therein. With respect to antisense oligonucleotides in particular, administration via peptide conjugation was among the exemplary methods of administration that had been described in the art. *See* specification at page 26, line 28, through page 27, line 10 and references cited and incorporated by reference therein. As noted in the specification, the intravenous administration of up to 700 mg of antisense oligonucleotide to a patient over

a course of 10 days (*i.e.*, 0.05 mg/kg/hr) was shown to produce no signs of toxicity. *See* specification at page 30, line 28, through page 31, line 9 and the reference cited and incorporated by reference therein.

Moreover, at the time of the effective filing date of the present application, several successful therapeutic applications of antisense oligonucleotides had been demonstrated. Examples include:

- Antisense oligonucleotides against protein kinase C alpha (PKC- $\alpha$ ) mRNA produced clinical responses when administered to ovarian cancer patients. *See Galderisi et al., J. Cell. Physiol. 181:251-257 (1999)*<sup>1</sup> at page 253, bottom right column; *see also Agrawal, Tibtech. 14:376-387 (1996)*<sup>2</sup> at page 376, top right column;
- Antisense oligonucleotides against c-raf mRNA produced "promising clinical response[s]" in patients with breast, prostate, and colon cancer. *See Galderisi* at page 254, top left column;
- Antisense oligonucleotides against bcr/abl mRNA, when administered to a patient with chronic myelogenous leukemia (CML), resulted in "complete hematological remission." *See Galderisi* at page 254, middle left column;

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<sup>1</sup> Galderisi was submitted as Exhibit A in the Reply Under 37 C.F.R. § 1.111 filed by Appellants on December 19, 2003, and is submitted herewith as Exhibit 1, in compliance with 37 C.F.R. § 41.37(c)(ix).

<sup>2</sup> Agrawal was cited by the Examiner at page 4 of the Office Action dated September 25, 2003, and is submitted herewith as Exhibit 2, in compliance with 37 C.F.R. § 41.37(c)(ix).

- Antisense oligonucleotides targeted against c-myc mRNA, when delivered into balloon-denuded porcine coronary arteries, caused a reduction in neointimal thickness (which is usually increased following balloon angioplasty). *See* Galderisi at page 254, right column;
- Antisense oligonucleotides directed against the 5'-region of the preS gene of duck hepatitis B virus (DHBV), injected intravenously into DHBV-infected ducks, inhibited DHBV replication and caused a decrease in serum DHBV DNA levels. *See* Galderisi at page 255, left column; *see also* Agrawal at page 376, paragraph bridging left and right columns. (Other antiviral applications of antisense oligonucleotides are described in Galderisi, at page 254, bottom right column, through page 255, bottom left column);
- Antisense oligonucleotides targeted to the RI $\alpha$  subunit of protein kinase A (PKA) in nude mice inhibited tumor growth. *See* Agrawal at page 376, top right column;
- Antisense oligonucleotides directed against angiotensin, administered via portal vein administration or direct injection to inbred spontaneously hypertensive rats, lowered blood pressure in the rats. *See* Agrawal at page 376, middle right column;
- Antisense oligonucleotides targeted to *c-myc*, intravenously administered to nude mice bearing a human melanoma explant, reduced tumor growth. *See* Agrawal at page 376, middle right column;



- Antisense oligonucleotides directed against the dopamine type-2 receptor gene, administered intracerebroventricularly, inhibited receptor expression, receptor mRNA levels and behavioral effects in animals. *See* Agrawal at page 376, middle right column;
- Antisense oligonucleotides directed against oxytocin mRNA, administered intracerebroventricularly to rats, blocked lactation. *See* Agrawal at page 376, middle right column; and
- Antisense oligonucleotides directed against the V1 vasopressin receptor, delivered to the brain septal area, reduced anxiety-related behavior in rats. *See* Agrawal at page 376, middle right column.

Finally, Galderisi provides a discussion of Vitravene, an antisense oligonucleotide approved for marketing in the United States and indicated for the local treatment of cytomegalovirus (CMV) retinitis in patients with AIDS. *See* Galderisi at page 255, paragraph bridging left and right columns. As noted by Galderisi, "Vitravene, which is based on an antisense mechanism and is commercially available in the United States, has shown that some substantial successes can be reached with the antisense technique." *See* Galderisi at page 255, middle right column.

The above-described examples illustrate clearly that antisense-based therapeutics were well established in the art and were proven to be clinically effective at the time of the effective filing date of the present application.

**4. *Level of Ordinary Skill***

The multiple examples of successful therapeutic applications of antisense oligonucleotides indicates that the level of skill possessed by those in the art of antisense-based therapeutics was very high at the time of the effective filing date of the present application. Persons of ordinary skill would therefore have had an expert level of understanding of, and experience in, the design, construction and administration of antisense oligonucleotides for a wide variety of therapeutic applications.

**5. *Level of Predictability in the Art***

**(a) *The Basic Aspects of Antisense Therapeutics Are Not Unpredictable***

The use of antisense molecules for therapeutic purposes is predictable. Designing and implementing effective antisense therapeutic approaches involves: (a) designing an appropriate antisense molecule (based on the selection of an appropriate target sequence); and (b) delivering antisense molecules to cells. *See Galderisi at page 251, bottom right column, through page 253, top left column.* Although various technical considerations are taken into account in selecting an antisense sequence and deciding upon an appropriate method of cellular delivery, the specification provides substantial guidance regarding these aspects. Furthermore, techniques were well known and available in the art that significantly enhanced the predictability of both of these aspects of antisense therapy.

**(i) Target Sequence Selection**

Selecting an appropriate target sequence for use in the context of the present invention would have been highly predictable in view of the claim language and the teachings in the specification. First, the claim specifies that the antisense oligonucleotide is complementary to an NTP mRNA sequence corresponding to nucleotides 150-1139 of SEQ ID NO:1. The specification provides exemplary regions of SEQ ID NO:1 to which the antisense oligonucleotides of the invention may be complementary, and the specification further provides exemplary oligonucleotide sequences (SEQ ID NOs:9, 10 and 11) that can be used with the methods of the invention. *See* specification at page 25, lines 18-28. The specification would have therefore directed one of ordinary skill in the art to regions within SEQ ID NO:1 that would serve as effective target sequences in the context of the claimed methods. Thus, based on the teachings in the specification alone, the selection of an appropriate target sequence would have been highly predictable.

In addition, computer modeling of target mRNA secondary structure was available in the art to facilitate the selection of sequences that are available for antisense recognition and binding. *See* Galderisi at page 252, bottom left column. According to Galderisi:

Modeling of the secondary structure of the target mRNA by computer software can be used for target selection of antisense molecules. Such a method carefully considers the potential folding pattern of a chosen mRNA as derived from its particular nucleotide sequence. After determining the free energy of a given secondary structure, the most probable folding structures are indicated, showing open loops and bulges that are accessible for oligonucleotides for efficient hybridization.

*See* Galderisi at page 252, bottom left column.

The effectiveness of computer-assisted modeling of mRNA secondary structure in defining antisense target sequences was well established prior to the effective filing date of the present application. *See, e.g., Jaroszewski et al., Antisense Res. Dev. 3:339-348 (1993) (abstract)*<sup>3</sup>. According to Jaroszewski, "[t]he model obtained by consideration of 30 lowest-energy computer-simulated structures is consistent with the high accessibility of the AUG initiation codon region known from digestion with nucleases and with previous antisense inhibition studies reported in the literature," and that, using additional antisense inhibition data, "the inhibitory activity of the oligonucleotides showed correlation with the calculated secondary structure of mRNA, in particular at low oligonucleotide-to-mRNA ratios (correlation coefficient, 0.95)." *Id.*

In addition, routine screening methods were available in the art that would have significantly improved the predictability of effective target sequence selection. *See* Section VII.B.7(c), below.

Thus, the first aspect of establishing an effective antisense therapy regimen, *i.e.*, selecting an appropriate target sequence, was very predictable in view of the availability of computer-assisted mRNA secondary structure software and routine screening methods.

#### **(ii) Cellular Delivery of Antisense Molecules**

The second major aspect of establishing an effective antisense therapy regimen, *i.e.*, the delivery of antisense molecules to cells, was also predictable at the time of the

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<sup>3</sup> The Jaroszewski abstract was submitted as Exhibit B in the Reply Under 37 C.F.R. § 1.111 filed by Appellants on December 19, 2003, and is submitted herewith as Exhibit 3, in compliance with 37 C.F.R. § 41.37(c)(ix)

effective filing date of the present application. Several strategies were well known and available in the art for increasing the efficiency of cellular delivery of antisense molecules. Examples include the use of liposomes, lipophilic carriers, cationic lipids, virions, modified donor cells and peptide conjugation. *See* specification at page 26, line 17, through page 27, line 10, and at page 32, line 4, through page 33, line 3, and the references cited and incorporated by reference therein; *see also* Galderisi at page 252, middle right column, through page 253, top right column.

Accordingly, the cellular delivery of antisense molecules would not have been regarded as unpredictable, from the standpoint of one of ordinary skill in the art.

***(b) The Examiner Has Not Established That The Field of Antisense Therapeutics is Unpredictable***

Much of the Examiner's rationale for the enablement rejection is based on the assertion that "[t]he art of nucleic acid based therapies is an unpredictable art." *See* Final Office Action, page 5. To support this assertion, the Examiner cited three references: Agrawal, *Tibtech.* 14:376-387 (1996), Branch, *TIBS* 23:45-50 (1998)<sup>4</sup>, and Jen and Gewirtz, *Stem Cells* 18:307-319 (2000)<sup>5</sup>. *See* Final Office Action, pages 5-8. As discussed below, none of these references support the contention that the field of antisense therapeutics is unpredictable.

***(i) Agrawal***

Agrawal is cited for the proposition that "[o]ligonucleotide must be taken up by cells in order to be effective." *See* Final Office Action at page 5 (quoting Agrawal at

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<sup>4</sup> Copy submitted herewith as Exhibit 4.

<sup>5</sup> Copy submitted herewith as Exhibit 5.

page 378, bottom left column). The specific portions of Agrawal cited by the Examiner, however, relate to the cellular uptake of oligonucleotides *in culture*. See Agrawal at page 378, bottom left column, through page 379, middle left column (under the heading "Cell culture system and target gene"). Agrawal nonetheless concludes that "[i]t is clear from some of the studies mentioned in this review and many other published reports that PS-oligonucleotides show more sequence-specific antisense activity in *animal models* than in cell culture experiments." Agrawal at page 384, middle right column (emphasis added).

The present invention relates to the administration of antisense oligonucleotides *to animals*, not to cells in culture. Thus, the concerns with cellular uptake of oligonucleotides in cell culture, as discussed in Agrawal, are irrelevant to the enablement of the claimed methods involved in this appeal. Importantly, Agrawal indicates that antisense technology *is* effective in animals. Thus, Agrawal does not support the contention that the field of the present invention (*i.e.*, the therapeutic application of antisense oligonucleotides *in animals*) is unpredictable.

**(ii) Branch**

The Examiner cited Branch, *TIBS* 23:45-50 (1998), for issues relating to "non-antisense effects" and accessibility of oligonucleotides to target RNA. See Final Office Action, pages 6-7. Branch, however, does not indicate that the field of antisense therapeutics is unpredictable.

1) *Non-Antisense Effects*

Branch does not suggest that "non-antisense effects" render the field of antisense-based therapeutics unpredictable. Branch notes that non-antisense effects "occur when a nucleic acid drug acts on some molecule other than its intended target." *See* Branch at page 45, middle column. Branch emphasizes, however, that non-antisense effects are often desirable in therapeutic settings and are problematic generally only in pure research applications. *See* Branch at page 46, left column. According to Branch:

Non-antisense effects pose a dilemma for the pharmaceutical industry. These effects include the stimulation of B-cell proliferation and the inhibition of viral entry into cells, responses *which are potentially useful*. Non-antisense ODNs [oligodeoxynucleotides] are already being developed as adjuvants to boost the efficacy of immunotherapies and vaccines. Phase III clinical trials of ISIS 2922, a phosphorothioate oligonucleotide (S-ODN) that induces both antisense and non-antisense effects, are also under way in patients with cytomegalovirus-associated retinitis. It is hoped that this compound's diverse mechanisms of action will yield a single drug that provides many of the benefits of combination therapy. However, as Anderson and colleagues have observed, *characteristics that are advantages in pharmaceutical drugs can be disadvantages in research reagents*. Thus, a safe and effective nucleic acid drug that slows the progression of AIDS would be of tremendous value, even if it were to act by inhibiting a perplexing combination of viral proteins rather than by binding to HIV RNA as originally intended. However, this same compound would be useless as an agent to selectively destroy HIV RNA, and could be ruinous if used in experiments of HIV molecular biology without knowledge of its mechanism of action.

Branch, page 46, left column (internal citations omitted, emphases added).

The present invention relates to the use of antisense molecules to treat animals, not for *in vitro* research purposes. Since, Branch makes it clear that non-antisense

effects may pose problems in research settings but are beneficial in clinical settings, Branch does not support the notion that the field of the present invention is unpredictable.

The discussion in Branch of non-antisense effects at best relates to the *mechanism* of how antisense molecules work; Branch does not suggest that antisense molecules are ineffective or unpredictable; Branch explicitly acknowledges that antisense molecules "can *undeniably* hit their intended targets." See Branch at page 45, bottom right column (emphasis added). Section 112, first paragraph does not require that an inventor know how or why his invention works. See, e.g., *Newman v. Quigg*, 877 F.2d 1575, 1581, 11 U.S.P.Q.2d 1340, 1345 (Fed. Cir. 1989). Thus, the discussion in Branch relating to non-antisense effects (which is essentially a discussion of *the mechanism* by which antisense molecules exert their effects) cannot be used to support a rejection for non-enablement.

## 2) *Oligonucleotide Accessibility*

With respect to oligonucleotide accessibility, Branch simply indicates that not all oligonucleotides are equal in their ability to bind to a particular RNA target, and that screening may be needed to identify optimal sequences. See Branch at page 49, left column. Branch does not, however, suggest that designing antisense oligonucleotides is unpredictable. To the contrary, Branch describes examples in which researchers successfully identified effective antisense oligonucleotides using routine screening methods. See Branch at page 49, left and center columns. See also Section VII.B.7(a),



below. Branch, therefore, does not support the assertion that the field of the invention is unpredictable.

**(iii) *Jen and Gewirtz***

Finally, the Examiner cited Jen and Gewirtz, *Stem Cells* 18:307-319 (2000), as indicating that "progress needs to be made in the art," and outlining the "key challenges" to the field. *See* Final Office Action, pages 7-8. The need for progress, and the existence of "challenges," however, does not indicate that antisense-based methods are necessarily unpredictable.

**6. *Amount of Direction Provided by Inventors / Working Examples***

**(a) *The Specification Provides More Than Adequate Direction To Practice the Claimed Methods***

The present specification provides more than adequate direction for persons of ordinary skill in the art to practice the claimed methods.

In terms of selecting an appropriate target sequence, the claims specify that the antisense oligonucleotide is complementary to an NTP mRNA sequence corresponding to nucleotides 150-1139 of SEQ ID NO:1. In addition, the specification provides exemplary regions of SEQ ID NO:1 to which the antisense oligonucleotides of the invention may be complementary. *See* specification at page 25, lines 18-24. The specification also notes that antisense oligonucleotides which are non-homologous to pancreatic thread protein (PTP) are preferred. *See* specification at page 26, lines 1-6. A person of ordinary skill in the art, in view of the teachings in the specification and the general knowledge in the art regarding antisense therapies, would have been able to

select appropriate nucleotide sequences to effectively interfere with the expression of AD7c-NTP in an animal using the claimed methods.

The specification also teaches exemplary modes of formulation and administration of antisense oligonucleotides for use in the practice of the claimed methods. *See* specification at page 30, lines 21-27. The specification teaches various routes of administration that can be used in the context of the present invention such as, *e.g.*, parenteral, subcutaneous, intravenous, intramuscular, intra-peritoneal, transdermal, intrathecal or intracranial. *See* specification at page 31, lines 1-3. Moreover, the specification recites various factors to be considered in determining the appropriate dosage of antisense oligonucleotides that are administered in the practice of the claimed methods. According to the specification:

The dosage administered will be dependent upon the age, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. For example, as much as 700 milligrams of antisense oligonucleotide has been administered intravenously to a patient over a course of 10 days (*i.e.*, 0.05 mg/kg/hour) without signs of toxicity (Sterling, "Systemic Antisense Treatment Reported," *Genetic Engineering News* 12(12):1, 28 (1992)).

Specification at page 31, lines 3-9. In view of the guidance provided in the specification, a person of ordinary skill in the art would have been able to prepare and effectively administer antisense oligonucleotides of the invention without undue experimentation.

**(b)      *The Examiner Has Not Established That The Amount of Guidance Provided in the Specification is Insufficient***

In explaining the rejection, the Examiner has made various statements relating to the amount of guidance provided in the present specification. As discussed below, the

Examiner's statements in this regard are either incorrect and/or do not take into account the knowledge possessed by persons of ordinary skill in the art.

First, the Examiner stated that:

The instant specification does not provide guidance or examples that would show by correlation what sequences of antisense based nucleic acid compounds of the method would predictably provide for treatment or prevention of disease in general or for the treatment of dementias of Alzheimer's type of neuronal degeneration specifically.

Final Office Action, page 3. This assertion, relating to antisense sequence selection, is incorrect and ignores the high level of knowledge and skill that was available in the art relating to the identification of antisense target sequences for therapeutic purposes.

As noted above, the claims specify that the antisense oligonucleotides used in the claimed methods are complementary to an NTP mRNA sequence corresponding to nucleotides 150-1139 of SEQ ID NO:1. The specification provides further guidance as to target sequences that can be selected within the AD7c-NTP coding sequence. *See* specification at page 25, lines 18-24, and at page 26, lines 1-6. Moreover, the specification provides exemplary oligonucleotide sequences (SEQ ID NOs:9, 10 and 11) that can be used with the methods of the invention. *See* specification at page 25, lines 26-28. The Examiner has not presented any evidence to suggest that practicing the claimed methods using antisense oligonucleotides targeted to the general regions and particular sequences of SEQ ID NO:1 identified in the specification would have involved anything more than the application of routine techniques.

In addition, computer based modeling programs (*see* Galderisi at page 252, bottom left column; Jaroszewski abstract), and routine screening methods (*see* Branch at page 49, left and center columns) would have been known and readily available to persons of ordinary skill in the art. *See* Section VII.B.5(a), above. Such methods, in light of the teachings in the specification, would have further aided in the identification of suitable antisense target sequences within nucleotides 150-1139 of SEQ ID NO:1.

Thus, from the perspective of one of ordinary skill in the art, the guidance provided in the specification for the identification of appropriate antisense target sequences is more than adequate to enable the practice of the claimed methods.

Second, the Examiner stated that:

The instant specification does not provide guidance or examples that would show by correlation what modes of delivery would predictable [sic] provide for a treatment of disease in general and for the treatment or prevention of dementias of Alzheimer's type of neuronal degeneration in particular. . . . The specification does not, for example, provide guidance on how to deliver antisense to neuronal cells . . .

Final Office Action, pages 3 and 4. These statements, regarding the guidance provided in the specification for delivering antisense molecules to neuronal cells, are incorrect.

The specification sets forth various exemplary methods by which antisense molecules of the invention can be delivered to neuronal cells for the treatment of dementias of the Alzheimer's type of neuronal degeneration. For instance, the specification indicates that AD7c-NTP antisense oligonucleotides can be delivered to neuronal cells using virions that may be introduced into the blood stream for delivery to

the brain, and by the use of modified donor cells (*e.g.*, fibroblast cells, neuronal cells, glial cells, and connective tissue cells) that are grafted into the central nervous system. *See* specification at page 32, line 4, through page 33, line 3 and the references cited and incorporated by reference therein. The specific technical details of such methods were well known in the art at the time of the effective filing date of the present application. *See id.*

Thus, from the perspective of one of ordinary skill in the art, the guidance provided in the specification for the delivery of antisense molecules to neuronal cells is more than adequate to enable the practice of the claimed methods.

Finally, the Examiner stated that:

The instant specification does not provide any examples of inhibiting AD7c-NTP in cells in culture or in an animal or provide guidance that would show by correlation the treatment or prevention of dementias of Alzheimer's type of neuronal degeneration via the administration of antisense based nucleic acid compounds.

Final Office Action, page 4.

Appellants note that working examples are not necessary to satisfy the enablement requirement. *See Gould v. Quigg*, 822 F.2d 1074, 1078 (Fed. Cir. 1987). A specification need not contain a working example if the invention is otherwise disclosed in such a manner that one skilled in the art would be able to practice the invention without undue experimentation. *See In re Borkowski*, 422 F.2d 904, 908 (CCPA 1970). The M.P.E.P. instructs that lack of a working example is a factor to be considered in

assessing enablement "*especially in a case involving an unpredictable and undeveloped art.*" See M.P.E.P. § 2164.02 (emphasis added).

As discussed above, the field of antisense-based therapeutics was neither an unpredictable nor an undeveloped art at the time of the effective filing date of the present application. See Section VII.B.3, above. For example, several successful therapeutic applications of antisense oligonucleotides had been demonstrated, and at least one antisense pharmaceutical composition (Vitravene) had been approved for marketing in the United States. See *Id.*; see also Galderisi at page 255, paragraph bridging left and right columns. Moreover, the specification provides ample teachings that, when combined with the knowledge possessed by those skilled in the art, would have fully enabled the practice of the claimed methods, regardless of the presence or absence of a working example in the specification.

**7. *Quantity of Experimentation Needed to Practice the Claimed Methods Based on the Content of the Disclosure***

In order to practice the claimed methods, a person of ordinary skill in the art would have needed to engage in, at most, a reasonable amount of routine experimentation. As explained above, designing and implementing an effective antisense therapeutic approach involves two basic aspects: (a) selecting an appropriate antisense target sequence; and (b) delivering the antisense molecules to cells. See Galderisi at page 251, bottom right column, through page 253, top left column. Neither aspect would have required an amount of experimentation that would be considered undue by one of ordinary skill in the art.

**(a) *Selecting Appropriate Target Sequences***

Only a modest amount of experimentation would have been needed to select a target sequence within SEQ ID NO:1 to which the antisense oligonucleotides of the invention are complementary, in order to effectively inhibit or decrease the expression of AD7c-NTP and thereby treat dementias of the Alzheimer's type of neuronal degeneration.

First, independent claim 35 specifies that the antisense oligonucleotide is complementary to an NTP mRNA sequence corresponding to nucleotides 150-1139 of SEQ ID NO:1. This alone would reduce the amount of experimentation needed to identify an acceptable target sequence by specifying the general region within SEQ ID NO:1 to which the antisense oligonucleotides are complementary. The amount of experimentation needed to identify an acceptable target sequence would be further reduced in view of the specification which teaches exemplary regions of SEQ ID NO:1 to which the antisense oligonucleotides of the invention may be complementary, and the particular exemplary antisense oligonucleotide sequences (SEQ ID NOs:9, 10 and 11) that can be used in the practice of the invention. *See* specification at page 25, lines 18-28.

Second, a person of ordinary skill in the art would have been able to further narrow down and identify likely effective target sequences (*e.g.*, sequences that are not blocked by secondary structures) through the use of computer modeling programs. At the time of the effective filing date of the present application, sequences identified by such programs had been shown to correlate with actual antisense inhibitory activity in

experimental systems. *See, e.g.,* Jaroszewski abstract; *see also* Galderisi at page 252, middle left column. Therefore, the use of computer-based secondary structure modeling programs would even further reduce the amount of experimentation needed to identify acceptable target sequences.

Third, in addition to, or as an alternative to computer modeling approaches, screening methods for identifying appropriate target sequences could have been used in the context of the present invention. Such methods were routine in the art and therefore would not have been regarded as undue experimentation. Examples of successfully used screening methods are described in Branch at page 49, left and middle columns.

For example, Branch describes an example in which antisense oligonucleotides that could bind to the structurally complex  $\beta$ -globin mRNA molecule were identified by *in vitro* screening of 1938 candidate oligonucleotides. *See* Branch at page 49, left column. In another example, an antisense oligonucleotide that was able to reduce the level of c-raf kinase mRNA by more than five-fold in cells was identified by screening 34 candidate oligonucleotides. *See* Branch at page 49, paragraph bridging left and center columns, and Fig. 3. It is therefore clear that persons of ordinary skill in the art typically engaged in such screening methods to identify effective antisense oligonucleotides. Screening methods to identify effective antisense target sequences would therefore not have been regarded as undue experimentation.

With respect to *in vitro* screening methods, the Examiner at page 7 of the Final Office Action quoted the following sentence from Branch: "It is not yet clear whether *in vitro* screening techniques . . . will identify ODNs that are effective *in vivo*." *See*



Branch, page 49, right column. This statement, however, does not indicate that such screening techniques would necessarily be ineffective or that they would be regarded as undue experimentation. As noted by Branch, "[i]f tests of 50 molecules identify good candidates, tests of thousands of compounds should identify better ones." Branch at page 49, right column. Thus, Branch acknowledges that "good" antisense molecules can be identified by screening 50 candidates and that "better" antisense molecules would likely be identified by screening thousands of candidates. There is no evidence of record to suggest that screening 50 or even thousands of antisense oligonucleotides would have been regarded as undue experimentation. Branch therefore supports Appellants' position that *in vitro* screening methods to identify effective antisense target sequences would not have been regarded as undue experimentation.

**(b) *Delivering Antisense Molecules to Neuronal Cells***

The second basic aspect relating to the practice of the claimed methods, *i.e.*, delivering antisense molecules to neuronal cells, would likewise not have required a large quantity of experimentation by persons skilled in the art. As discussed in Sections VII.B.5(a) and VII.B.6(b), above, methods for enhancing the cellular delivery of antisense molecules, and methods for delivering polynucleotides to neuronal cells in general, were well known and available in the art at the time of the effective filing date of the present invention. In fact, Agrawal describes at least two examples of the successful administration of antisense molecules for the treatment of neuronal defects (antisense oligonucleotides directed against the dopamine type-2 receptor gene, administered intracerebroventricularly, inhibited behavioral effects in animals, and antisense oligonucleotides directed against the V1 vasopressin receptor, delivered to the

brain septal area, reduced anxiety-related behavior in rats. *See* Agrawal, page 376, middle right column). Thus, with the aid of the various available methods and strategies in the art, the amount of experimentation needed to effectively deliver antisense molecules to neuronal cells in animals would have been minimal.

**8. *The Numerous Examples in the Art of Successful Antisense Therapies Demonstrates That the Practice of the Claimed Methods Would Not Have Required Undue Experimentation***

Appellants wish to emphasize that, at the time of the effective filing date of the present application, there were numerous examples in the art of the successful application of antisense strategies for therapeutic purposes. *See* Section VII.B.3, above. The ability of others to successfully employ antisense oligonucleotides to produce a variety of positive clinical outcomes demonstrates that antisense therapies in general did not involve undue experimentation. No evidence has been presented to explain why antisense therapies of the present invention would have required undue experimentation when, clearly, others were able to use antisense therapies in other contexts *without* undue experimentation.

The Examiner has dismissed Appellants' arguments regarding the many examples in the art of the successful application of antisense therapies. According to the Examiner, the cited examples allegedly involve the use of "specific methodologies using specific antisense oligonucleotides that have undergone extensive characterization and experimentation before their use in the treatments described." *See* Final Office Action at page 8. The Examiner, however, has not cited anything to support this assertion. For example, the Examiner has not provided any basis for the assertion that the

oligonucleotides used in the examples cited by Appellants had "undergone extensive characterization and experimentation" before being successfully used.

Moreover, the proper legal standard is "undue" experimentation not "extensive" experimentation. (Experimentation, even complex experimentation, is not undue if the art typically engages in such experimentation. *See Massachusetts Institute of Technology v. A.B. Fortia*, 774 F.2d 1104 (Fed. Cir. 1985)). The Examiner has not argued or presented any evidence to indicate that the amount of experimentation that was involved in the development of the antisense molecules used in the examples cited by Appellants would have been regarded as undue by persons of ordinary skill in the art. In fact, since there are so many examples in the art in which antisense therapies were successfully used, it is difficult to fathom how the amount of experimentation needed to practice these methods could be regarded as undue. *See id.*

In addition, the Examiner's argument of non-enablement is based solely on the assertion that the use of antisense therapies *in general* is unpredictable. *See, e.g.*, Final Office Action, page 5. It is inconsistent and contradictory for the Examiner to, on the one hand, assert that antisense therapies in general are unpredictable, and on the other hand, reject Appellants' arguments and evidence of the successful use of antisense therapies simply because they relate to "general techniques." *See* Office Action, page 8. The Examiner has not provided any specific evidence or scientific reasoning to explain why the general techniques and methods used in the multiple examples cited by Appellants (in which antisense oligonucleotides produced positive clinical outcomes) could not have also been used successfully in the practice of the claimed methods. Thus,

Appellants believe that the Examiner has not given sufficient consideration to the multiple examples of the successful use of antisense therapies in the art which indicate that the presently claimed methods could have likewise been practiced without undue experimentation.

### 9. *Summary of Enablement Analysis*

A proper analysis of the enablement factors set forth in *Wands* indicates that practicing the full scope of the claimed methods would not have required undue experimentation. The specification and the knowledge possessed by persons of ordinary skill in the art would have fully enabled the claimed methods.

At the time of the filing date of the present application, the scientific field relating to the therapeutic administration of antisense oligonucleotides was well developed and predictable. There were several examples from the scientific literature which showed the successful use of antisense oligonucleotides to treat a variety of diseases and conditions including cancer, viral infection, high blood pressure, arterial neointimal thickening following balloon angioplasty, and neurological-based behavioral defects. Various methods and techniques were known and available in the art for selecting effective antisense target sequences and for delivering antisense molecules to cells. The development and administration of antisense-based therapeutics to treat dementias of the Alzheimer's type of neuronal degeneration would therefore *not* have required undue experimentation.

Moreover, Appellants submit that the Examiner has not established a *prima facie* case of non-enablement. The Examiner has not presented evidence which would indicate

or suggest that practicing the methods of the present invention would have required undue experimentation. The Examiner has focused on alleged insufficiencies in the specification but has failed to acknowledge the substantial amount of information and skills that were possessed by persons of ordinary skill in the art; such information and skills would have supplemented the teachings of the specification. The Examiner has also failed to adequately address the fact that several others in the art had successfully employed antisense strategies to produce positive clinical outcomes for a variety of disorders. The Examiner has not explained why similar positive outcomes would not have been expected to be achieved by the practice of the present invention.

The Examiner has cited three references to support the assertion that the field of antisense base therapeutics is unpredictable<sup>6</sup>. The cited references, however, merely highlight certain technical considerations to be addressed in optimizing antisense-based therapeutics. In addition, each of the cited references actually acknowledges that antisense-based methods have been successfully used to treat certain disorders. The references therefore do not support the enablement rejection but actually support Appellants' contention that the practice of the currently claimed methods would not have required undue experimentation. Accordingly, the Examiner has not established a *prima facie* case of non-enablement.

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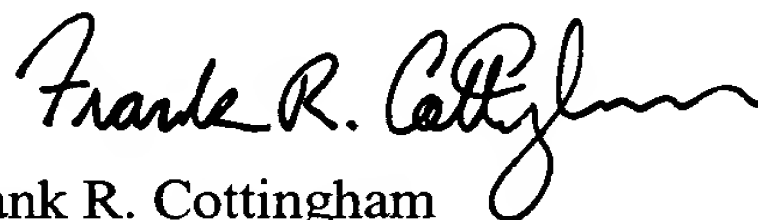
<sup>6</sup> Appellants note that the Examiner's treatment of the cited references amounts to nothing more than the citation of various sentences and sentence fragments that have been taken out of context in an attempt to support the enablement rejection. The Examiner has not presented a careful analysis of the totality of these references and has failed to acknowledge the portions of these references that support the enablement of the present invention.

**C. Conclusion**

In view of the foregoing discussion, Appellants submit that the subject matter defined by claims 35 and 37-42 is fully enabled and that the Examiner has not met his burden of establishing a *prima facie* case of non-enablement. Accordingly, Applicants respectfully request that the Board reverse the Examiner's 35 U.S.C. § 112, first paragraph rejection of claims 35 and 37-42 and remand this application for issue.

Respectfully submitted,

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***VIII. Claims Appendix***

35. A method for the treatment of dementias of the Alzheimer's type of neuronal degeneration, said method comprising administering to an animal in need thereof an antisense oligonucleotide which is complementary to an NTP mRNA sequence corresponding to nucleotides 150-1139 of SEQ ID NO:1.

37. The method of claim 35, wherein said antisense oligonucleotide is a 15 to 40 mer.

38. The method of claim 35, wherein said antisense oligonucleotide is selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10 and SEQ ID NO:11.

39. The method of claim 35, wherein said antisense oligonucleotide is deoxyribonucleic acid.

40. The method of claim 35, wherein said antisense oligonucleotide is a deoxyribonucleic acid phosphorothioate.

41. The method of claim 35, wherein said antisense oligonucleotide is a derivative of a deoxyribonucleic acid or a derivative of a deoxyribonucleic acid phosphorothioate.

42. The method of claim 35, wherein said antisense oligonucleotide is administered to said animal as part of a pharmaceutically acceptable carrier.



***IX. Evidence Appendix***

- Exhibit 1 Galderisi, U., et al., "Antisense Oligonucleotides as Therapeutic Agents," J. Cell. Physiol. 181:251-257 (1999).
- Exhibit 2 Agrawal, S., "Antisense Oligonucleotides: Towards Clinical Trials," Tibtech. 14:376-387 (1996).
- Exhibit 3 Jaroszewski, J.W., et al., "Targeting of Antisense DNA: Comparison of Activity of Anti-Rabbit Beta-Globin Oligodeoxyribonucleotide Phosphorothioates with Computer Predictions of mRNA Folding," Antisense Res. Dev. 3:339-348 (1993) (abstract).
- Exhibit 4 Branch, A.D., "A Good Antisense Molecule is Hard to Find," TIBS 23:45-50 (1998).
- Exhibit 5 Jen, K-Y and Gewirtz, A.M., "Suppression of Gene Expression by Targeted Disruption of Messenger RNA: Available Options and Current Strategies," Stem Cells 18:307-319 (2000).

***X. Related Proceedings Appendix***

No decisions have been rendered by a court or the Board in any proceedings identified in the *Related Appeals and Interferences* section (Section II).

# Antisense Oligonucleotides as Therapeutic Agents

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Antisense oligonucleotides can block the expression of specific target genes involved in the development of human diseases. Therapeutic applications of antisense techniques are currently under investigation in many different fields. The use of antisense molecules to modify gene expression is variable in its efficacy and reliability, raising objections about their use as therapeutic agents. However, preliminary results of several clinical studies demonstrated the safety and to some extent the efficacy of antisense oligodeoxynucleotides (ODNs) in patients with malignant diseases. Clinical response was observed in some patients suffering from ovarian cancer who were treated with antisense targeted against the gene encoding for the protein kinase C- $\alpha$ . Some hematological diseases treated with antisense oligos targeted against the bcr/abl and the bcl2 mRNAs have shown promising clinical response. Antisense therapy has been useful in the treatment of cardiovascular disorders such as restenosis after angioplasty, vascular bypass graft occlusion, and transplant coronary vasculopathy. Antisense oligonucleotides also have shown promise as antiviral agents. Several investigators are performing trials with oligonucleotides targeted against the human immunodeficiency virus-1 (HIV-1) and hepatitis viruses. Phosphorothioate ODNs now have reached phase I and II in clinical trials for the treatment of cancer and viral infections, so far demonstrating an acceptable safety and pharmacokinetic profile for continuing their development. The new drug Vitravene, based on a phosphorothioate oligonucleotide designed to inhibit the human cytomegalovirus (CMV), promises that some substantial successes can be reached with the antisense technique. *J. Cell. Physiol.* 181:251-257, 1999. © 1999 Wiley-Liss, Inc.

The use of oligonucleotides as selective inhibitors of gene expression offers a rational approach for the prevention and treatment of some gene-mediated disorders. In the antisense approach, oligonucleotides block the expression of specific target genes involved in the development of the pathological processes. Therapeutic applications of antisense technique currently are under investigation in many different fields, including oncology, hematopathology, cardiovascular diseases, and infectious diseases (Agrawal and Iyer, 1995; Wagner, 1995; Agrawal, 1996; Crooke and Bennet, 1996; Bradbury, 1997; Wagner and Flanagan, 1997; Agrawal and Zhao, 1998).

Antisense oligodeoxynucleotides (ODNs) are short stretches of DNA (12-30 nucleotides) that are complementary to a target mRNA. The ODNs selectively hybridize to their complementary RNA by Watson-Crick base pairing rules. The translation of target mRNA is inhibited by an active and/or a passive mechanism when hybridization occurs between the complementary helices. Passive mechanism results from the hybridization between the mRNA and exogenous nucleotide sequence, which leads to duplex formation that prevents the ribosomal complex from reading the message (Fig. 1A). In the active mechanism, hybridization allows for binding of RNaseH,

which destroys the RNA but leaves the DNA oligonucleotide intact to hybridize with yet another mRNA target (Fig. 1B; Wagner and Flanagan, 1997; Bradbury, 1997; Monia, 1997; Kronenwett and Haas, 1998b).

The concept of antisense technology is simple. However, the development of antisense oligonucleotides as broadly applicable therapeutic agents has been slow and difficult (Stein and Cheng, 1993; Stein and Krieg, 1994; Stein, 1995; Bradbury, 1997; Wagner and Flanagan, 1997; Romano et al., 1998a).

## SELECTION OF A SPECIFIC AND EFFECTIVE ANTISENSE MOLECULE

### Selection of target sequence

The selection of an appropriate target sequence is the first step in the process of drug development. In

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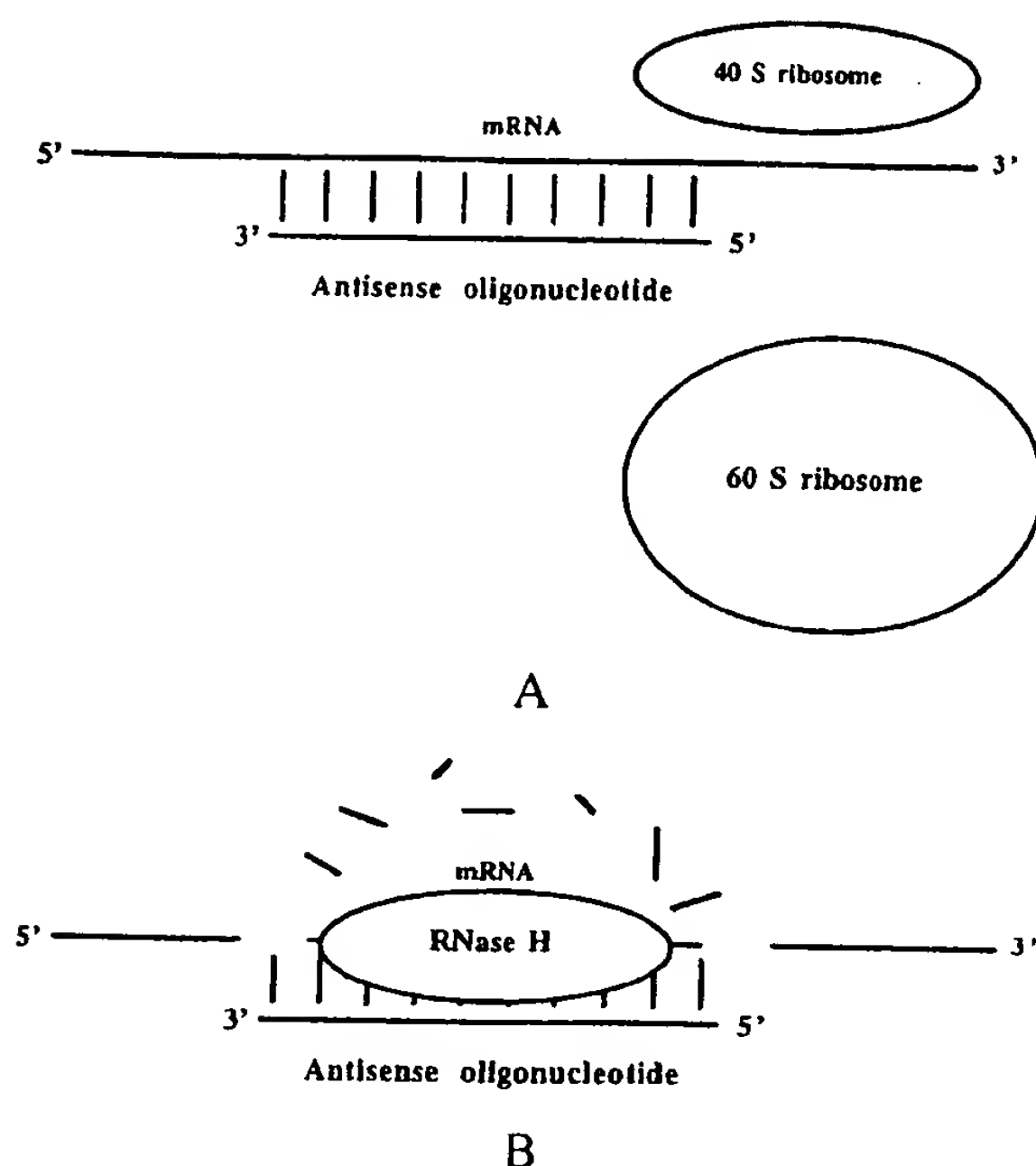


Fig. 1. Mechanism of action of antisense oligonucleotides. In the passive mechanism (A), the translation of target mRNA is inhibited by the hybridization between the mRNA and exogenous nucleotide sequence. This leads to duplex formation that prevents the ribosomal complex from reading along the message. In the active mechanism (B), the mRNA-ODN heteroduplex forms a substrate for RNase H, an enzyme that recognizes and selectively destroys the RNA portion of the mRNA-ODN hybrid.

fact, the hybridization between antisense oligos and the target sequence, which has a particular three-dimensional structure resulting from secondary and tertiary structures, depends on the accessibility of the target sequence. Only small stretches of mRNA sequence, devoid of interchain hybridization, are available for heteroduplex formation with DNA oligonucleotides which affects the activity of ODNs. For example, only one of 34 ODNs targeting human *c-ras* mRNA demonstrated potent antisense activity. Modeling of the secondary structure of the target mRNA by computer software can be used for target selection of antisense molecules. Such a method carefully considers the potential folding pattern of a chosen mRNA as derived from its particular nucleotide sequence. After determining the free energy of a given secondary structure, the most probable folding structures are indicated, showing open loops and bulges that are accessible for oligonucleotides for efficient hybridization (Stein and Krieg, 1994; Monia, 1997; Wagner and Flanagan, 1997; Agrawal and Zhao, 1998).

#### Selection of chemical modifications

Cells contain a variety of exo and endonucleases that can degrade ODNs. A number of nucleotide and nucleoside modifications have made the oligonucleotide more resistant to nuclease digestion than the native ODNs that have phosphodiester linkages in their nu-

cleotide backbone. Oligonucleotides that have been modified to enhance their nuclease resistance survive intact for longer times than unmodified oligonucleotides. A variety of oligonucleotide modifications have enhanced or conferred nuclease resistance, thus allowing oligos to reach their intracellular targets (Capaccioli et al., 1993; Gewirtz, 1993; Agrawal and Iyer, 1995; Galderisi et al., 1999). Phosphorothioates are one of the most frequent variants of ODNs. One of the oxygens in the phosphate backbone in these molecules is replaced by a sulfur atom (Fig. 2). Increased protection against cleavage by both exonucleases and endonucleases is the result of such chemical modification. Other modifications give rise to methylphosphonates, in which a methyl group is substituted for an oxygen of phosphate; other modifications are phosphoramidates that show an amide linkage inserted instead of an ester bridge; peptide nucleic acid, having the phosphate sugar backbone substituted by an alkylamide linkage (Fig. 2; Eckstein, 1983; Henry et al., 1997b; Flanagan, 1998; Galderisi et al., 1999). The oligonucleotides also may be "chimeric oligonucleotides." Chimeric oligonucleotides contain two or more chemically distinct regions. These molecules are designed to confer more than one beneficial property to ODNs, such as increased nuclease resistance, increased uptake into cells, or increased binding affinity for the RNA target. At present, phosphorothioate oligos are the most widely used molecules in cell cultures, animals, and humans (Agrawal and Iyer, 1995; Monia, 1997; Agrawal and Zhao, 1997; Shinozuka et al., 1997; Galderisi et al., 1999).

#### Cellular delivery of ODNs

The main problem in increasing the bioavailability of administered ODNs is the protection against cleavage. While the mechanism involved in the cellular ODN uptake still is not clear, there also is a great variation between different cell types with regard to their ability to internalize oligo molecules. A receptor-mediated endocytosis seems to play a main role in ODN uptake, followed by the release of ODNs from endocytotic vesicles into the cytoplasm (Loke et al., 1989; Iversen et al., 1992; Bennett et al., 1994; Beltinger et al., 1995; Kronenwett and Haas, 1998b).

The cellular internalization of ODNs is not efficient in *in vitro* models, hence, many techniques have been used to enhance ODN uptake. The most widely used method is based on cationic lipids. These molecules form complexes with the anionic nucleic acids and protect them against degradation. The macromolecular complexes have a positive charge at the surface, allowing binding to cell membrane, which is negatively charged. Following attachment to the membrane, the complexes are taken up via endocytosis. Additional improvement in oligo uptake can be anticipated. For example, efforts are being made to modify liposomal lipids by adding ligands of cellular receptors as well as antibodies directed against antigens expressed on the respective target cells (Bennet et al., 1992; Capaccioli et al., 1993; Stein and Krieg, 1994; Beltinger et al., 1995; Gokhale et al., 1997; Kronenwett and Haas, 1998b).

While direct administration of ODNs *in vitro* is not an effective delivery method, phosphorothioate ODNs

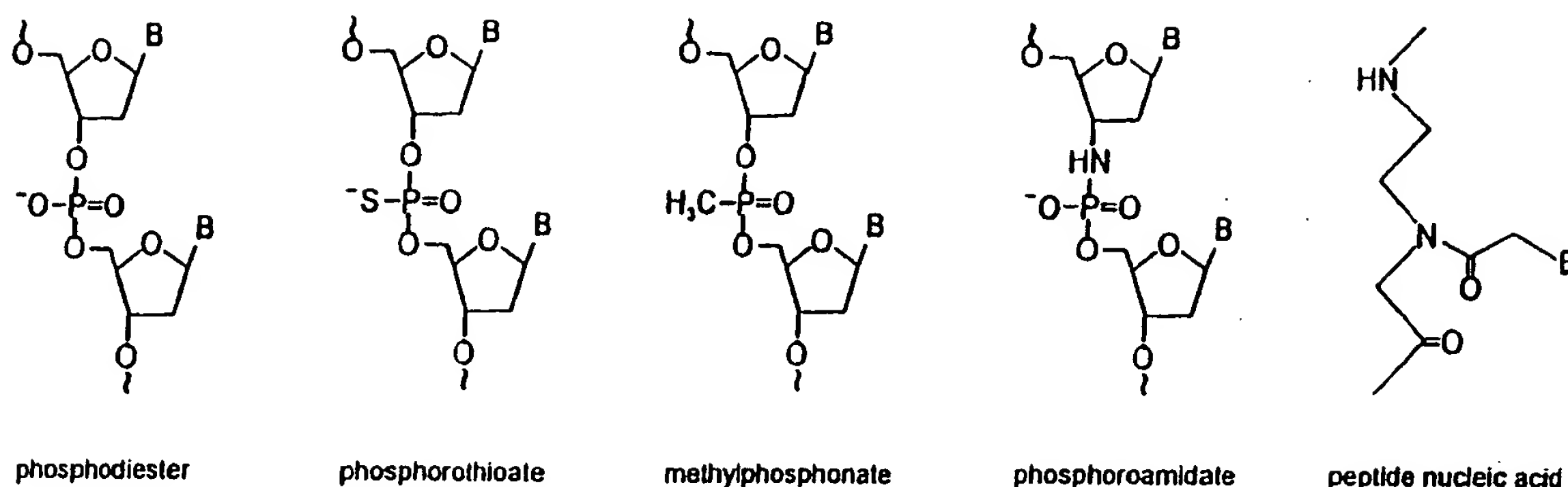


Fig. 2. Chemical structure of internucleotide linkages of unmodified (phosphodiester) and modified oligonucleotides. B, nucleotide bases.

administered intravenously without any delivery reagent to animal models showed effective and specific antisense inhibition. These surprising results helped revive antisense technology and encouraged researchers to move to clinical trials (Wagner, 1995; Crooke and Bennet, 1996; Geary et al., 1997).

#### Antisense oligonucleotide evaluation

The aim of antisense researchers is to show down-regulation of a target gene in a sequence-specific manner, while control ODNs, which are oligonucleotides not complementary to the chosen target mRNA, should show little or no downregulation capability. However, several examples of nonsequence-specific effects have been seen with ODNs, particularly with the chemically modified molecules. Oligonucleotides, which are negatively charged, can interact with positively charged molecules. For example, ODNs can bind in a sequence-independent manner the gp120 protein of the human immunodeficiency virus-1 (HIV-1), bovine serum albumin, the receptor for platelet-derived growth factor, the receptor for basic fibroblast growth factor, and several other cellular proteins (Stein and Cheng, 1993; Stein and Krieg, 1994; Stein, 1995; Crooke and Bennet, 1996).

Antisense side effects also could be due to sequence-specific interactions between ODNs and cellular proteins that can cause the so-called "sequence-dependent but nonantisense effect." For example, the presence of four contiguous guanosine residues in an ODN, the G quartet, can result in an antiproliferative effect regardless of the remaining sequence of the molecule (Crooke and Bennet, 1996; Stein and Krieg, 1994; Vaerman et al., 1995; Wagner, 1995).

#### ANTISENSE THERAPY

The idea of antisense-mediated gene inhibition therapy is as fascinating as other types of gene therapy (Romano et al., 1998a; Giordano et al., 1998). The following examples suggest that these compounds may have some therapeutic efficacy likely through a combination of antisense and nonsequence-dependent effects on gene function.

#### Pharmacokinetics

Several experiments assessing the pharmacokinetics and toxicology of ODNs have been performed in mice, rats, and monkeys (Cossum et al., 1993; Galbraith et al., 1994; Iversen et al., 1995; Zhang et al., 1995; Leeds et al., 1998). The pharmacokinetics were independent of the length as well as of the sequence of ODNs. When injected intravenously or intraperitoneally, the nucleic acids were excreted mainly in the urine within 24 h. However, detectable levels were found in most tissues except the brain for up to 48 h, with only 15–50% degradation for phosphorothioate ODNs. In monkeys and in phase I clinical trials, dose-dependent hypotension, complement activation, and transient prolongation of thromboplastin time were observed as side effects. Preliminary results of other clinical studies demonstrated the safety and, to some extent, the efficacy of antisense ODNs in patients with malignant diseases (Iversen et al., 1995; Glover et al., 1997; Henry et al., 1997a; Raynaud et al., 1997; Sereni et al., 1999).

#### Antisense clinical trials for cancer treatment

A major signal transduction pathway involving the enzyme protein kinase C (PKC) has a critical influence on cell proliferation and differentiation (Liu and Heckman, 1998). An increased expression of PKC- $\alpha$  is found in many human cancers including those of the breast and colon and in brain tumors. Inhibition of human PKC- $\alpha$  gene expression has occurred with antisense ODNs both in vitro and in vivo (Dean et al., 1996; Zhang et al., 1997). A phosphorothioate ODN, directed against the 3'-untranslated region of PKC- $\alpha$ , has been tested by ISIS Pharmaceutical (Carlsbad, CA) and Novartis (Basel, Switzerland) in some human tumor cell lines grown in athymic mice. This oligo, named ISIS3521, was administered intravenously once a day for 14 days and showed a noticeable tumor growth decrease in T-24 bladder carcinoma, in A-549 non-small cell lung carcinoma, and in Colo 205 colon carcinoma xenograft models with a 50% inhibitory dose between 60 and 600  $\mu\text{g/kg}$  per day. After this success, ODN entered a phase I clinical trial. Clinical responses were observed in 3 of 17 treated patients, all having ovarian cancer (McGraw et al., 1997; Flanagan, 1998).



The c-raf gene codes for a highly conserved serine-threonine-specific protein kinase (Magnuson et al., 1994; Kerkhoff and Rapp, 1998; Yuryev and Wennagle, 1998). Certain abnormal proliferative conditions are associated with raf expression and therefore are believed to be responsive to inhibition of raf expression (Worland et al., 1990). Examples of abnormal proliferative conditions are hyperproliferative disorders such as cancers, hyperplasias, pulmonary fibrosis, and angiogenesis (Nakatsu et al., 1986; Pfeifer et al., 1989; Naumann et al., 1997).

A phosphorothioate ODN named ISIS5132, which is complementary to c-raf mRNA, has shown a strong sequence-specific inhibition of c-raf gene expression in some subcutaneously implanted human tumor cell lines in nude mice. Subsequently, phase I clinical trials have demonstrated the safety of this ODN. Furthermore, several patients with breast, prostate, and colon cancers showed promising clinical response. Based on this data, phase II clinical trials were initiated in 1998 (Monia et al., 1996; Henry et al., 1997b; McGraw et al., 1997; Monia, 1997; Flanagan, 1998; Monteith et al., 1998).

The ODNs may also be useful for ex vivo bone marrow purging, a method used for treatment of patients suffering from leukemias and lymphomas. Large amounts of bone marrow can be surgically extracted from patients and stored in vitro, while the patients receive conventional treatment. Following relapse, the patients can be rescued by reinfusion of their own bone marrow cells that have been "purged" of residual malignant cells employing ODNs targeted against altered gene expression associated with the leukemias and/or lymphomas (De Fabritiis et al., 1998).

Bcr/abl mRNA is the product of a neo-gene created by a reciprocal translocation involving the c-abl and the bcr genes. The expression of the bcr/abl oncogene is involved in the pathogenesis of chronic myelogenous leukemia (CML). Some clinical experience with bcr/abl targeted antisense ODNs in CML has been reported (Vaerman et al., 1995; Skorski et al., 1997; Kronenwett and Haas, 1998a). De Fabritiis and colleagues (1998) treated a patient with CML in an accelerated phase with autologous bone marrow transplantation. Before reinfusion, cells were purged in vitro with a 26-mer phosphorothioate ODN targeted against bcr/abl mRNA. The patient was reported to be in complete hematological remission (Gewirtz, 1993; De Fabritiis et al., 1998).

The expression of the bcl2 gene, which is involved in the apoptosis pathway, is overregulated in most non-Hodgkin lymphomas (Reed et al., 1990; Tsurusawa et al., 1998; Reed, 1998). Genta (San Diego, CA) has developed an antisense ODN targeted against bcl2 mRNA. This oligo results in a complete remission in nude mice inoculated with human follicular lymphoma cells. Based on these results, phase I trials have been initiated (Raynaud et al., 1997; Webb et al., 1997; Flanagan, 1998; Kronenwett and Haas, 1998b; Bloem and Lockhorst, 1999; Chaudhary et al., 1999).

#### **Antisense ODN as potential drugs in other human diseases**

Antisense therapy is emerging as a potential agent for the treatment of cardiovascular diseases such as

restenosis after angioplasty, vascular bypass graft occlusion, and transplant coronary vasculopathy. The local transfer of antisense molecules into the vascular wall offers a promising alternative for the treatment of atherosclerosis-related diseases at the cellular and molecular levels. Blood vessels are among the easiest targets for this gene therapy technique because in such conditions as postangioplasty restenosis, only a transient inhibition of target gene expression is required (Shi et al., 1994; Laitinen and Yla-Herttuala, 1998).

Coronary balloon angioplasty is a procedure in which a catheter bearing an inflatable distal balloon is inserted into the arterial lumen and expanded. The method is used to open stenotic regions in vessels closed by arterial plaques and fatty deposits. This technique is used often for patients suffering from atherosclerosis. Smooth muscle cell (SMC) proliferation of the vascular wall is a normal response to several pathophysiological stimuli, including those associated with procedures for mechanically opening stenoses. If the proliferation is extensive, restenosis could follow the procedure. In particular, as many as 50% of the patients undergoing successful coronary angioplasty can develop recurrent coronary artery obstructions. Several different classes of pharmacological agents have been employed to inhibit SMC proliferation but as yet, unsuccessfully.

One approach is to inhibit mitogens that act on the cell surface of SMCs. The c-myc gene product is encoded by an immediate-early response gene, the expression of which can be induced by various mitogens. C-myc expression is involved in the signal transduction pathways leading to cell division. Studies have demonstrated that proliferating cells have higher levels of c-myc mRNA and protein than do quiescent cells (Paggi et al., 1996; Nesbit et al., 1999).

Several investigators have demonstrated the in vitro growth-inhibitory effect of antisense oligomers targeting the c-myc proto-oncogene in human SMCs (Bennett et al., 1994; Shi et al., 1994). These in vitro studies provided the rationale for assessing c-myc antisense oligomers in the prevention of neointima in vivo. For this purpose, antisense oligomers targeted against c-myc mRNA were delivered into balloon-denuded porcine coronary arteries. Despite rapid plasma clearance following local delivery, oligomers persisted at the site of injection for at least 3 days, exceeding by severalfold their concentration in peripheral organs. The morphometric analyses, carried out 1 month after transcatheter c-myc antisense oligomer administration, showed a significant reduction in maximal neointimal thickness in the antisense-treated group compared with controls. These changes in vascular remodeling following denuding injury resulted in an increase in the residual lumen in the antisense-treated animals. Since c-myc antisense oligomers reduced the formation of neointima in denuded coronary arteries, a potential therapeutic use for the prevention of coronary restenosis can be hypothesized (Shi et al., 1994; Mannion et al., 1998).

The use of antisense oligonucleotides has also emerged as a powerful new approach as antiviral agents (Selvam et al., 1996; Caselmann et al., 1997; Lima et al., 1997; Wagner and Flanagan, 1997; Veal et al., 1998). In fact, the initial therapeutic applications of ODN were supposed to be as an antiviral agent. Ste-

phenson and Zamecnik (1978) disclosed antisense oligonucleotides as inhibitors of Rous sarcoma virus replication in chicken fibroblasts.

HIV is responsible for the disease that has come to be known as acquired immune deficiency syndrome (AIDS). The HIV genome tends to mutate at a high rate, causing great genetic variation between strains of the virus and between virus particles of a single infected individual. Therapeutic agents currently used in the treatment of AIDS often cause severe side effects that preclude their use in many patients (Zhang et al., 1995; Junker et al., 1997; Romano et al., 1998b; Sereni et al., 1999).

One method for inhibiting specific gene expression that is believed to have promise is the antisense approach. Inhibition of viral gene expression and replication can be efficiently achieved by targeting the conserved sites of the viral RNAs that signal the synthesis of conserved HIV proteins, particularly the p24 core antigen protein. Some research groups have synthesized 20 mer/15 mer sequences targeted against the p24 core protein region of HIV. Initial clinical trials are based on ODN systemic administration (intravenously). Dosages that can be used for systemic administration preferably range from about 0.01 to 50 mg/kg administered once or twice per day. Evaluations of ODN activity are under examination (Zhang et al., 1995; Junker et al., 1997; Sereni et al., 1999).

Chronic infection with the hepatitis B virus (HBV) is a major health problem worldwide. The only established treatment is interferon with an efficacy of only 30–40% in highly selected patients. The discovery of animal viruses closely related to the HBV has contributed to active research on antiviral therapy of chronic HBV infection. The animal model tested and described by several authors are Peking ducks infected with the duck HBV (DHBV; Shinozuka et al., 1997; Soni et al., 1998; Xin and Wang, 1998). Molecular therapeutic strategies are based on antisense ODNs directed against the 5'-region of the preS gene of DHBV that inhibited viral replication and gene expression in vitro in primary duck hepatocytes. The in vivo studies showed that intravenous injection of antisense ODNs entrapped within liposomes enhances delivery of the ODNs to the liver and inhibits DHBV replication. Serum DHBV DNA levels fall rapidly, with a corresponding decrease in intrahepatic viral replicative intermediates at the end of the 5-day study period. These results demonstrate a potential clinical use for antisense DNA as antiviral therapeutic agents (Caselmann et al., 1997; Lima et al., 1997; Offensperger et al., 1998; Soni et al., 1998).

### THE FIRST ANTISENSE-BASED DRUG

Vitravene is the first in a class of novel therapeutic agents based on an antisense mechanism that has been approved for marketing in the United States. Vitravene is indicated for the local treatment of cytomegalovirus (CMV) retinitis in patients with AIDS who are intolerant to or have a contraindication to other treatments for CMV retinitis or who were insufficiently responsive to previous treatments (from www.vitravene.com). Vitravene consists of a phosphorothioate oligonucleotide designed to inhibit human CMV replication by an antisense mechanism. It has been shown in vitro to

inhibit replication of human CMV with a greater potency than either ganciclovir or foscarnet. It does not interfere with the antiviral activity of the anti-HIV drugs AZT and dideoxycytidine and it can be additive to the use of ganciclovir and foscarnet. Vitravene was equally potent against 21 independent clinical human CMV isolates, including several that were resistant to ganciclovir, foscarnet, and/or cidofovir (from www.vitravene.com).

### FUTURE PROSPECTS

The use of antisense to modify gene expression is variable in both its efficacy and reliability, which caused objections about its use as a therapeutic agent. Most of these concerns can be overcome by the development of a new generation of antisense molecules with improved target specificity and enhanced delivery to the target cells. However, one concept must be borne in mind, an oligonucleotide need not be exclusively complementary to its target nucleic acid sequence to be specific. An oligonucleotide is specifically hybridizable when binding of the oligonucleotide to the target interferes with the normal function of the target molecule sufficient to cause a loss of function. There is a sufficient degree of complementarity to avoid nonspecific binding of the oligonucleotide to nontarget sequences (Stein and Krieg, 1994; Stein, 1995; Monia, 1997).

Antisense ODNs already have shown their effectiveness in several preclinical studies. Phosphorothioate ODNs have reached phase I and II in clinical trials for the treatment of cancer and viral infections and have demonstrated an acceptable safety and pharmacokinetic profile for continuing their development. The new drug Vitravene, which is based on an antisense mechanism and is commercially available in the United States, has shown that some substantial successes can be reached with the antisense technique.

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# Antisense oligonucleotides: towards clinical trials

udhir Agrawal

Antisense oligonucleotides have the ability to selectively block disease-causing genes, thereby inhibiting production of disease-associated proteins. The specificity and application of antisense oligonucleotides have been strongly validated in animal models for various disease targets. Based on the pharmacological, pharmacodynamic and pharmacokinetic profiles, the first generation of antisense oligonucleotides – phosphorothioates – have reached the stage of human clinical trials for various diseases. While ongoing human clinical trials are being carried out to further establishing the safety and efficacy of these oligonucleotides, the experience gained is providing a basis for designing a second generation of antisense oligonucleotides.

There are two crucial parameters in drug design: the first is the identification of an appropriate target in the disease process, and the second is finding an appropriate molecule that has specific recognition and affinity for the target, thereby interfering in the disease process. Most of the targets employed for drug interaction are proteins, enzymes or hormones. The majority of the drugs discovered to date recognize their targets by mechanisms that are not well defined. The knowledge gained by this drug discovery process cannot be generalized for rational drug design.

In 1978, Zamecnik and Stephenson showed that replication of Rous sarcoma virus (RSV) can be inhibited by a synthetic 13mer oligodeoxynucleotide complementary to a specific mRNA of RSV (Ref. 1). As these authors predicted, this was the foundation of a novel approach to drug design – the antisense oligonucleotide approach<sup>2,3</sup> – that has two advantages over conventional drugs: (1) the mRNA of the disease target gene has a defined sequence, and (2) the antisense oligonucleotide interacts with the target gene by Watson–Crick base pairing, providing specificity and affinity. The use of antisense oligonucleotides to regulate specific gene expression allows one to apply the principles learned from working with one gene target to an indefinite number of genes, thereby providing therapies for many diseases.

Over the past 2–3 years, many reports have appeared in the literature confirming the application of antisense technology in *in vivo* models: intravenous administration of an antisense phosphorothioate oligonucleotide (PS-oligonucleotide) for ten days in ducks infected with duck hepatitis B virus almost eliminated the viral

DNA from the liver<sup>4</sup>; a single subcutaneous injection of antisense PS-oligonucleotide targeted to RI<sub>α</sub> subunit of protein kinase A (PKA) in nude mice inhibited tumor growth<sup>5</sup>; intraperitoneal administration in SK-1 hairless mice for seven days of an antisense PS-oligonucleotide to PKC- $\alpha$  reduced the levels of PKC- $\alpha$  mRNA in the liver<sup>6</sup>; in inbred spontaneously hypertensive rats, administration to the liver via the portal vein, or by direct injection, of an antisense oligonucleotide to angiotensinogen was shown to lower blood pressure<sup>7</sup>; intravenous administration of a PS-oligonucleotide targeted to *c-myc* reduced tumor growth in nude mice bearing a human melanoma explant<sup>8</sup>; intracerebroventricular administration of an oligonucleotide selectively inhibited dopamine type-2 receptor expression, dopamine type-2 receptor RNA levels and behavioral effects in animals<sup>9</sup>; intracerebroventricular administration for three days in rats of an end-capped antisense oligonucleotide to oxytocin mRNA blocked lactation<sup>10</sup>; and, delivery of an antisense oligonucleotide to V1 vasopressin receptor into the brain septal area reduced anxiety-related behavior in rats<sup>11</sup>. While the effects observed in these studies have been claimed to be sequence-specific, a few studies [e.g. inhibition of melanoma growth in severe combined immunodeficient (SCID)–human mouse model<sup>12</sup>] have demonstrated sequence-independent inhibition by PS-oligonucleotides *in vivo*.

In the studies mentioned above, the dose and route of administration, and the choice of oligonucleotide and gene target varied, but the observed effects were mostly sequence-specific. These studies indicate that:

- oligonucleotides remain stable following intravenous, intraperitoneal, subcutaneous, or intracerebroventricular administration;
- oligonucleotides are taken up by cells and tissues;

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- antisense oligonucleotides are effective at regulating gene expression by binding to the target gene mRNA; and
- oligonucleotides may also inhibit gene expression by mechanisms other than binding to the target gene mRNA.

#### Antisense oligonucleotides in cell culture models

*In vitro* (cell culture) studies with PS-oligonucleotides have shown both sequence-specific and sequence-independent inhibition of gene expression. The activity of an oligonucleotide in cell culture depends on many factors, including chemical modifications, the cell culture model and the sequence of the oligonucleotide.

#### Chemical modification of oligonucleotides

Chemical modification of oligonucleotides is one of the important parameters that is responsible for activity in cell culture models. PS-oligonucleotides, in which one of the non-bridging oxygens of the internucleotide phosphodiester linkages is replaced with sulfur (Fig. 1), have been studied extensively in various cell culture studies. In general, PS-oligonu-

cleotides are synthesized and evaluated as diastereomeric mixtures, which have potentially different biochemical and biophysical properties from their pure stereoregular counterparts. Stereoregular Rp PS-oligonucleotide<sup>13</sup> has a higher  $T_m$  (melting point), is a better substrate for RNase H, but is less resistant to nuclease degradation, than synthetic PS-oligonucleotides, whereas stereoregular Sp PS-oligonucleotide<sup>14</sup> has a lower  $T_m$  and is more resistant to nucleases than are synthetic PS-oligonucleotides; no data have yet been generated for the biological activity of stereoregular PS-oligonucleotides.

Inhibition of gene expression by PS-oligonucleotides occurs as a result of hybridization arrest (i.e. interference with the processing of mRNA by hybridization) and cleavage of the mRNA by RNase H (Ref. 15). However, because of their polyanionic nature, PS-oligonucleotides are able to bind to various factors, which may be the cause of non-sequence specific activity (Fig. 2). While the majority of studies use PS-oligonucleotides, limited studies have been carried out using other analogs of oligonucleotides in cell culture. These analogs of oligodeoxynucleotides include, for example, methylphosphonates<sup>16,17</sup>,

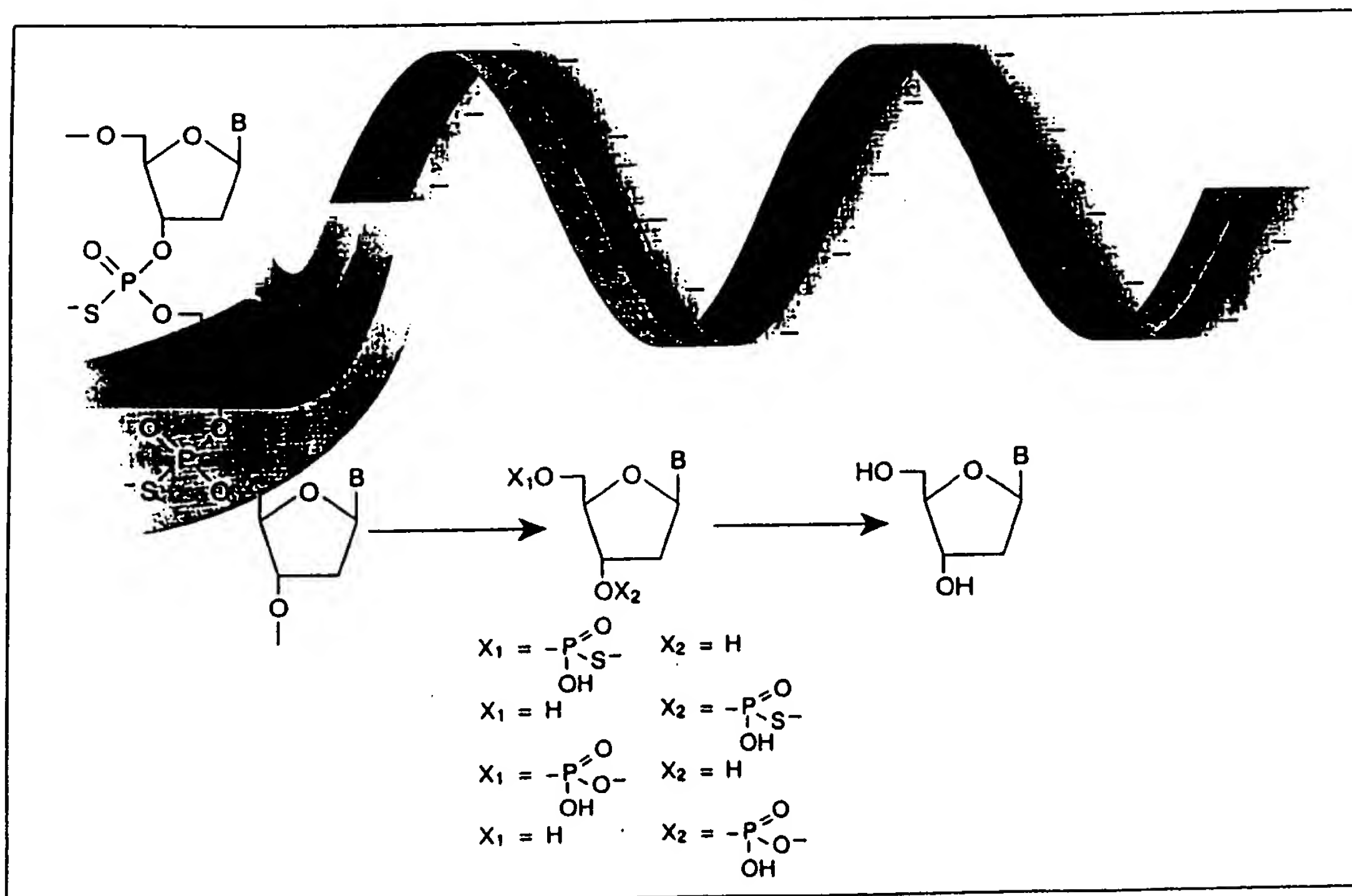


Figure 1

Structure of a PS-oligonucleotide. In an oligonucleotide of a defined sequence, various properties may depend on sequences (heterocyclic bases), sugar or phosphorothioate linkages (polyanionic). The metabolism of a PS-oligonucleotide may generate nucleotides, depending on digestion by 3' or 5' exonucleases and the mechanism of digestion, and nucleosides, along with shorter fragments of oligonucleotides. Metabolism of 1  $\mu$ mol of 25mer oligonucleotide will generate approximately 25  $\mu$ mol of nucleotides or 1  $\mu$ mol of each specific nucleotide and, ultimately, nucleosides. In cell culture studies, these metabolites are generated both intracellularly and extracellularly, and they remain present throughout the experiment. Oligonucleotides containing modified nucleosides following degradation will generate modified nucleotides or nucleosides. These modified nucleotides or nucleosides may exert more non-sequence-specific activity in addition to increasing the toxicity *in vivo*.

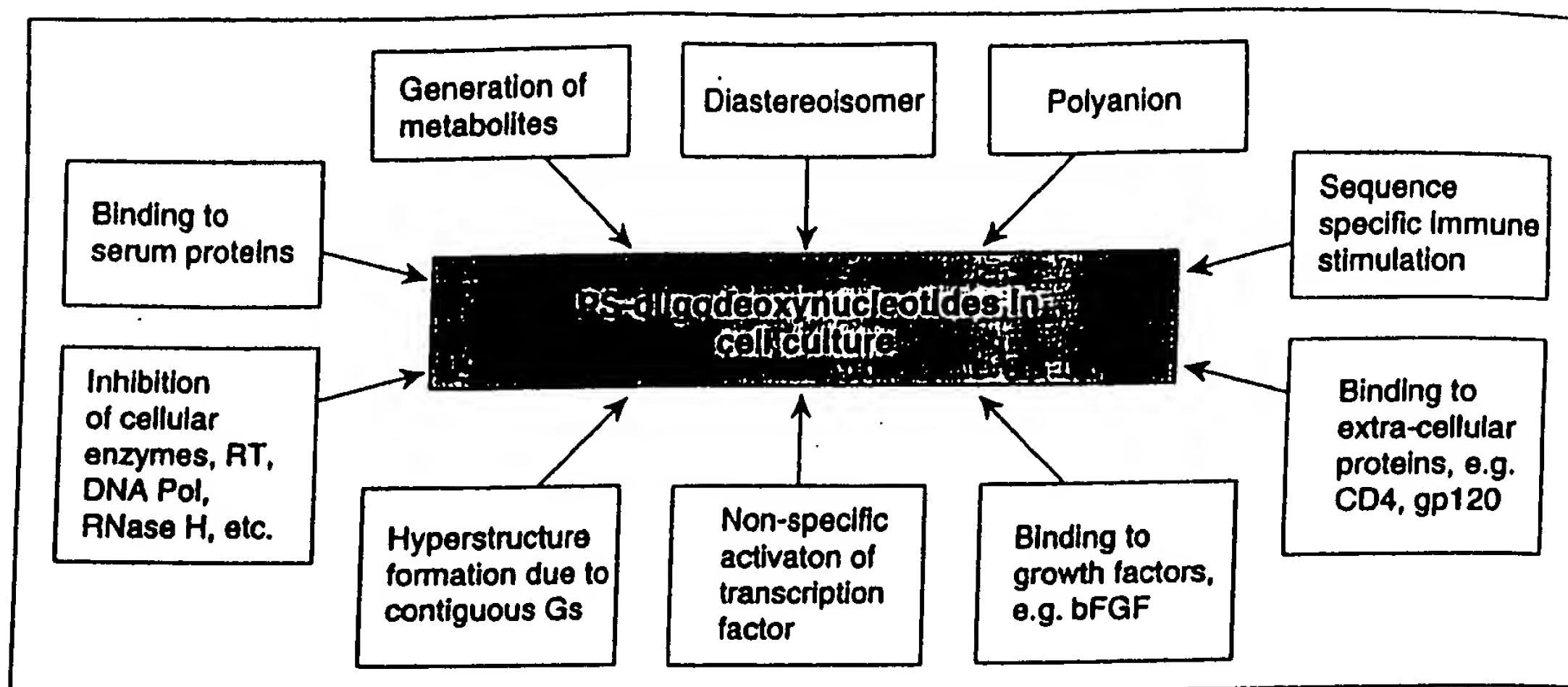


Figure 2

Some of the properties of PS-oligonucleotides, which may affect its antisense activity in cell culture systems. These properties may not have significant *in vivo* effects, because the local concentration of oligonucleotide and its metabolites remain low as a result of elimination and excretion. Abbreviations: bFGF, fibroblast growth factor b; DNA Pol, DNA polymerase; RT, reverse transcriptase.

phosphoramidates<sup>18</sup>, phosphorodithioates<sup>19</sup>, N3'→P5'-phosphoramidates<sup>20</sup> and oligoribonucleotide phosphorothioates<sup>21</sup>, and their 2'-O-alkyl analogs<sup>22-24</sup> and 2'-O-methylribonucleotide methylphosphonates<sup>25</sup>. These oligonucleotide analogs differ from each other in respect to their affinity for RNA, their stability to nucleases and the extent to which they activate RNase H. However, based on their biological activity, it is clear that oligonucleotide modifications that combine nuclease stability, specificity and affinity for mRNA, and that enable RNase H activation, may be more effective than modifications that lack any one of these properties.

Over the past few years, many novel oligonucleotide modifications that display interesting biophysical and biochemical properties have been documented<sup>26</sup>, but their biological activities in cell culture models have not yet been reported. Certainly, the biological activity of the newer analogs of oligonucleotides, either alone, or in combinations (see below), will provide further insight into the mechanisms of antisense oligonucleotides. In addition, modifications to oligonucleotides should not only be considered to improve the biological activity in cell culture experiments, but also to improve their pharmacokinetic and pharmacodynamic profile (Box 1).

#### Cell culture system and target gene

Oligonucleotide must be taken up by cells in order to be effective. Several reports have shown that efficient cellular uptake of oligonucleotides occurs in a variety of cell lines<sup>27-30</sup>, including primary cells<sup>31</sup>, whereas other reports indicate negligible cellular uptake of oligonucleotides<sup>32</sup>. Cellular uptake of oligonucleotides is a complex process; it depends on many factors, including the cell type, the stage of the cell cycle, the concentration of serum present in a biological medium, chemical modifications, and the

structure of the oligonucleotide, temperature, and incubation time. Variations in any of these parameters significantly affect the cellular uptake, intracellular localization and stability of the oligonucleotide. It is, therefore, difficult to generalize that all oligonucleotides are taken up in all cells with the same efficiency.

Antisense oligonucleotides are active in various cell culture studies from subnanomolar to micromolar concentrations. The potency of the oligonucleotide may vary depending on the time of addition of oligonucleotide to the system; this is particularly true in studies on the inhibition of virus replication. Oligonucleotides added to cells prior to infection may demonstrate different potency and specificity than if they were added to cells after infection<sup>33</sup>. The potency of an oligonucleotide may also vary depending on

#### Box 1. Properties which may be improved in second generation of oligonucleotides

##### Biological properties

- Affinity
- RNase H substrate
- Interaction with proteins/enzymes/cellular factors
- Cellular pharmacokinetics

##### Pharmacokinetic properties

- *In vivo* stability
- Clearance of metabolites
- Site-specific delivery
- Plasma half-life

##### Pharmacodynamic properties

- Partial thromboplastin time (aPTT)
- Complement activation
- Levels of transaminases
- Thrombocytopenia
- Splenomegaly



whether the cells are exposed to oligonucleotide alone or in combination with carriers (e.g. liposomes)<sup>34-36</sup>, or whether they are administered by microinjection<sup>32</sup>. Microinjection or using lipid carriers to supply an oligonucleotide increases the potency of the oligonucleotide in cell culture, but it is not clear how relevant this approach is for *in vivo* situations.

Another parameter that affects the potency and specificity of an oligonucleotide is the target gene itself. Some cell culture studies have been carried out using transfected cells, in which a segment of the gene of interest is inserted into a plasmid under the transcriptional control of an appropriate promoter<sup>37-39</sup>. In such studies, the rate of transcription, folding of RNA and the overall environment of the entire process is very different from the transcription and folding of the full-length gene of interest. In addition, the segment of the gene of interest alone may or may not have a crucial function. Any antisense activity observed in such artificial systems should be scrutinized carefully with respect to the disease process and its applicability to *in vivo* applications.

Antisense activity in cell culture may also be affected by factors, such as binding of oligonucleotides to cellular enzymes, cell-surface receptors, basic proteins and serum proteins (Fig. 2). In cell culture, the oligonucleotides are present in the extracellular medium throughout the duration of the experiment. The oligonucleotide is metabolized slowly to produce various metabolites, including shorter oligonucleotides, nucleotides and nucleosides, which are not eliminated from the culture medium, and may contribute to some of the non-sequence-specific effects. *In vivo*, however, the oligonucleotide and its metabolites are eliminated over a period of time, and the resulting lower local concentrations may reduce non-sequence-specific effects.

#### Effects of sequence of oligonucleotides

The antisense activity of an oligonucleotide depends on its length, base composition, sequence and chemical modifications. Some of these properties are responsible for the oligonucleotide's linear or secondary structures (e.g. hyperstructures), its affinity for the target sequence, the specificity of hybridization, RNase H activation, cellular uptake, *in vivo* stability, its interaction with cellular enzymes and cell-surface proteins, and other relevant characteristics. These properties, either alone or collectively, have a significant impact on the sequence-specific and sequence-independent antisense activity of oligonucleotides.

The effects of PS-oligonucleotides on various gene targets have been studied extensively in cell culture. To confirm whether these effects are sequence-specific, various investigators have used different types of control oligonucleotides, including reverse sequences, antisense sequences containing one or more mismatches, and random sequences containing the same base composition; we have also used a mixture of 4<sup>24</sup> oligonucleotide sequences as a control<sup>28</sup>. Various degrees of inhibition of gene expression have been

observed by mismatched/random or control oligonucleotides, although at higher concentrations than for antisense oligonucleotides. The inhibition of gene expression by so-called 'control oligonucleotides' is a phenomenon that depends on the nature of the oligonucleotide, i.e. its base composition, sequence and/or nature of the backbone. Many studies have shown that PS-oligonucleotides containing at least four contiguous Gs have inhibitory activity that is both sequence-specific and sequence-independent<sup>40-42</sup>.

In general, oligonucleotides containing four contiguous Gs form hyperstructures<sup>43</sup>. While this phenomenon has been observed with PS-oligonucleotides, the formation of the hyperstructure also depends on the nature of the flanking sequence (Fig. 3). PS-oligonucleotides with four contiguous Gs form dimeric and tetrameric hyperstructures in equilibrium with single-stranded structures. These hyperstructures are highly polyanionic and have increased stability towards nucleases, which may have a significant impact on the antisense activity of oligonucleotides and their specificity. It has also been shown that oligonucleotides containing certain palindromes may induce an immune response<sup>44</sup>, interferon production<sup>45</sup>, or natural killer cell activity<sup>46</sup>, all of which may also affect the outcome of the results.

#### Pharmacokinetics, tissue distribution and metabolism of oligonucleotides in animals

Pharmacokinetics, tissue distribution and *in vivo* stability studies of several PS-oligonucleotides of varying length and base composition have been performed. PS-oligonucleotides bind to serum proteins<sup>47,48</sup> in a species-dependent manner. Studies on the binding of a 25mer PS-oligonucleotide (GEM®91) to rat, rabbit, guinea pig and human serum show that binding to serum proteins is saturable, and the order of binding is: guinea pig>rat>rabbit>human<sup>49</sup>. Serum binding of PS-oligonucleotides may have some impact on pharmacokinetic profile and *in vivo* stability. Pharmacokinetics and tissue disposition of PS-oligonucleotides of varying length and base composition have been reported in mice<sup>50,51</sup>, rats<sup>52-54</sup> and monkeys<sup>55,56</sup> following intravenous<sup>50-56</sup>, intraperitoneal<sup>50</sup>, subcutaneous<sup>55</sup> and intradermal<sup>57</sup> administration. Distribution of PS-oligonucleotides following intravenous administration is very rapid: we have reported half-lives of distribution of <1 hour<sup>50,52,55</sup>, and similar data have been reported by others<sup>51,53,54,56</sup>. Plasma clearance is biphasic, with a terminal elimination half-life ranging from 40 to 60 hours<sup>50-56</sup> based on radioactivity levels (Fig. 4). Analysis of radioactivity extracted from plasma up to a 12 to 24-hour time point shows the presence of intact oligonucleotide along with metabolites<sup>50,52</sup>.

PS-oligonucleotides distribute to highly perfused organs – kidney, liver, bone marrow and spleen accumulate more of the administered dose than do other tissues (Fig. 4) – and the rate of absorption and elimination from different organs varies<sup>52</sup>. The liver accumulates most of the oligonucleotide, with the area under the curve (AUC;  $\mu\text{g ml}^{-1} \text{ hr}^{-1}$ ) for plasma and

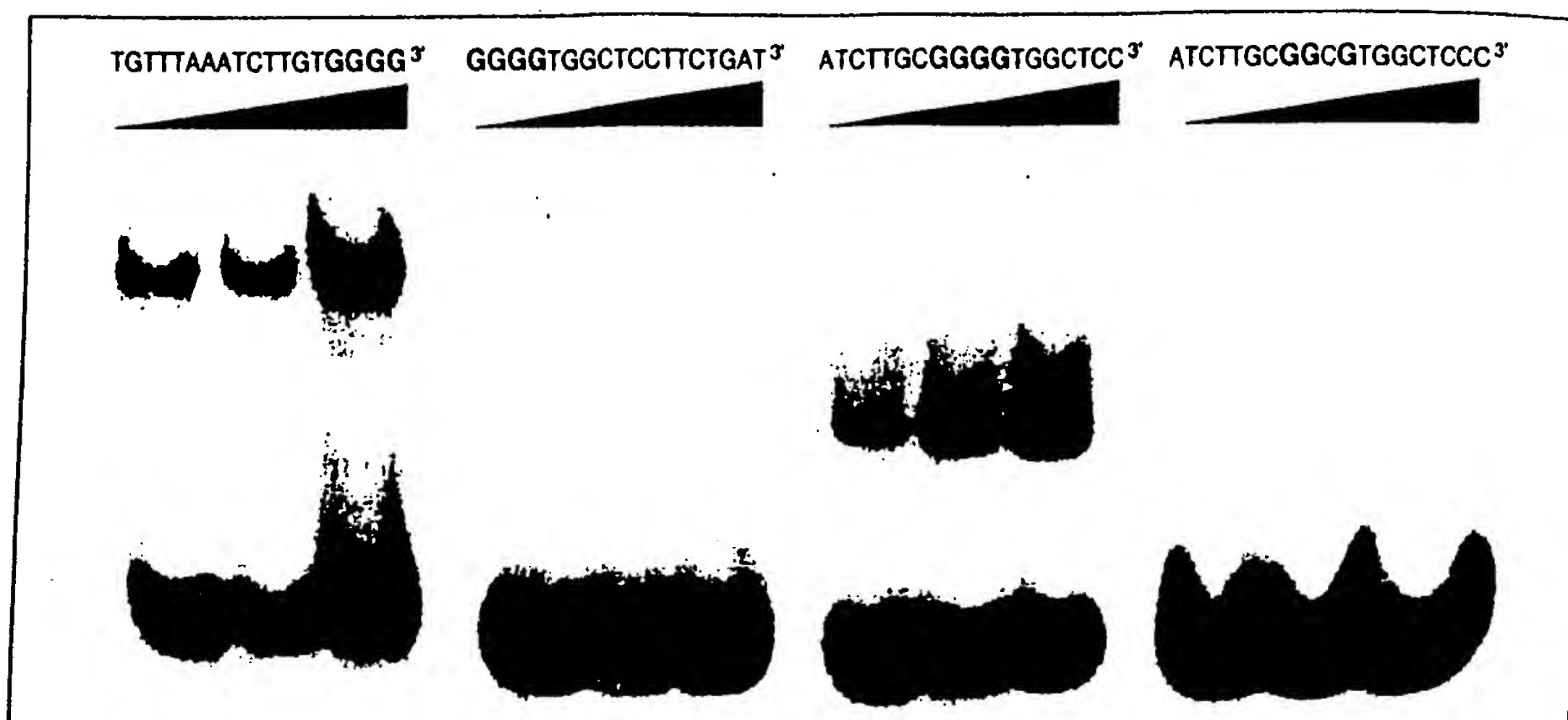


Figure 3

Ability of PS-oligonucleotides containing four Gs to form hyperstructures. Four 18mer PS-oligonucleotides were synthesized and purified by a similar procedure. Purified product was  $^{32}\text{P}$ -labeled at the 5' end, heated to  $95^\circ\text{C}$  in 110 mM sodium acetate and 40 mM potassium acetate (pH 7.6), and allowed to cool slowly at room temperature. Samples were analyzed on a 10% denaturing polyacrylamide gel. The gel shows 60, 120 and 185  $\mu\text{M}$  concentrations of each oligonucleotide. PS-oligonucleotides containing four Gs at the 3' end and in the middle demonstrate the formation of a hyperstructure, which is less mobile on the gel. The ability of PS-oligonucleotide containing four Gs at the 5' end to form hyperstructures under these conditions is very weak. The ability to form hyperstructures is disrupted by incorporation of a nucleoside other than G.

liver being 1432.51 and 6538.18, respectively, following intravenous administration of a  $30\text{ mg kg}^{-1}$  dose of PS-oligonucleotides in rats<sup>52</sup>. PS-oligonucleotides also accumulate in, and are retained by, the kidney<sup>50-56</sup>. Detailed studies of the distribution of PS-oligonucleotides in kidneys have shown that PS-oligonucleotides are present within Bowman's capsule, the proximal tubule lumen and within tubular epithelial cells<sup>58</sup>. The presence of PS-oligonucleotides in renal proximal tubules has recently been shown to suppress the renal  $\text{Na}^+/\text{P}_i$  co-transporter in a sequence-specific manner following intravenous administration in rats<sup>59</sup>. In the perfused rat model, co-injection of excess dextran sulfate with oligonucleotide resulted in a 40% reduction in renal uptake, suggesting that oligonucleotides may be taken up by the glomerular endothelial cells via a receptor-mediated mechanism<sup>60</sup>.

#### Site of administration

Subcutaneous administration of PS-oligonucleotides produced a profile of plasma clearance very similar to that for the intravenous route, except for the observation that the peak plasma concentration ( $C_{\text{max}}$ ) was lower (Fig. 5a), and the bioavailability of PS-oligonucleotides was about 85% (Ref. 55); similar results (84% bioavailability) have been reported following subcutaneous administration using a 24mer PS-oligonucleotide in mice<sup>56</sup>. Intraperitoneal administration shows a pharmacokinetic profile similar to the one observed for the subcutaneous route<sup>50</sup>, and similar profiles of plasma absorption and clearance have been observed for the following intradermal administration in rats<sup>57</sup>; bioavailability is about 65%, and slow metab-

olism of oligonucleotide occurs at the injection site. Intravitreal administration of a  $^{14}\text{C}$ -labeled PS-oligonucleotide shows that the elimination half-lives of the oligonucleotide from the vitreous humor and retina are 60 hours and 96 hours, respectively<sup>61</sup>. Once absorbed from the site of injection following subcutaneous, intradermal, or intraperitoneal administration, the pharmacokinetic profile and tissue disposition are similar to those following intravenous administration.

#### Oligonucleotide clearance and elimination

PS-oligonucleotides are primarily eliminated in urine, and to a lesser extent in feces (Fig. 4b). As much as 40% of the PS-oligonucleotide is eliminated in urine in 24 hours, and up to 60% is eliminated in 240 hours<sup>50,52,54,56</sup>. The majority of the oligonucleotide excreted in urine is in a degraded form. The rate of clearance in urine depends on the dose administered and the route of administration. At higher doses ( $\geq 30\text{ mg kg}^{-1}$ ), some intact oligonucleotide can be detected in urine, possibly as a result of saturation of protein binding<sup>50</sup>. Fecal excretion is a minor pathway of elimination of PS-oligonucleotides<sup>50,52</sup>. It is not yet clearly established whether clearance of the oligonucleotide is by metabolism (by exo- and endonucleases), by the reticuloendothelial (RE) system (a system shown to clear polyanionic molecules<sup>62,63</sup>) by binding to proteins/enzymes, or other factors, including varying rates of efflux from various tissues. In studies carried out in our laboratory with modified PS-oligonucleotides (PS-oligonucleotides containing a segment of 2'-O-methylribonucleotides or a segment of methylphosphonate linkages at both the 3' and 5'

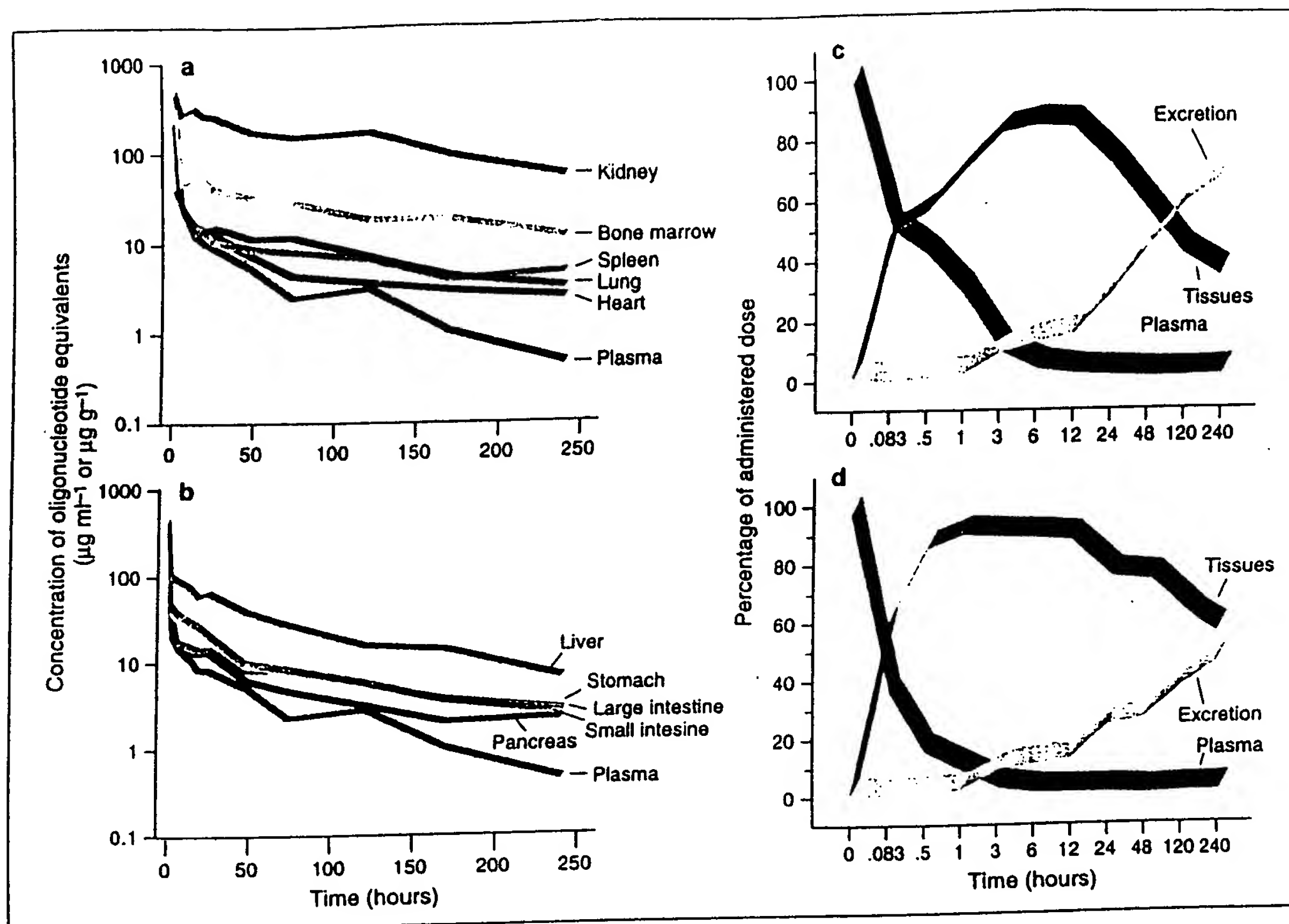


Figure 4

Plasma clearance, tissue disposition and excretion of PS-oligonucleotide. Following intravenous administration, PS-oligonucleotide is distributed from plasma to various tissues quite rapidly. The concentration of PS-oligonucleotide in most of the tissues is higher than in plasma six hours after administration. (a), (b) Show the disposition of oligonucleotide in certain tissues; details have been published elsewhere<sup>52</sup>. (c) Shows the combined concentration in all tissues, indicating that tissues absorb and retain oligonucleotide compared with the plasma, and elimination in urine is slower. (d) PS-oligonucleotides that have been modified to increase *in vivo* stability show more retention in tissues and slow clearance in urine. The details of this modified PS-oligonucleotide have been published elsewhere<sup>64</sup>.

ends), which shows significantly increased *in vivo* stability, the elimination in urine is almost half that for PS-oligonucleotides, suggesting that metabolism is one of the factors responsible for clearance<sup>64,65</sup>. This finding has also been detailed in a recent paper, in which 2'-O-propoxyoligoribonucleotide phosphorothioate, which shows increased *in vivo* stability, was eliminated in urine to a lesser degree than was PS-oligonucleotide<sup>66</sup>. This increased *in vivo* stability of modified PS-oligonucleotides may be advantageous for local administration (e.g. intraocular or intracerebroventricular) in cases in which modified PS-oligonucleotides may have long residence times following administration thereby requiring less frequent dosing. No studies have indicated whether the RE system is involved in clearance of PS-oligonucleotides, however, but the toxicity profile of the RE system suggests that this system is involved in clearance.

#### Studies in monkeys

There are fewer reports on the pharmacokinetics of PS-oligonucleotides in monkeys than in mice and

rats, but the monkey studies generally confirm what has been observed in these other systems. The initial and terminal elimination half-lives in plasma are between 0.6 and 1 hour, and 42.2 and 56.3 hours, respectively, following intravenous administration of PS-oligonucleotides<sup>55</sup>. The peak plasma concentration depends on the dose administered<sup>55</sup>, and the percentage of the dose excreted in urine is also dose dependent – about 27% and 53% is excreted in urine over 96 hours following administration of 1 and 5 mg kg<sup>-1</sup>, respectively<sup>55</sup>. In another study, the pharmacokinetic profile of a PS-oligonucleotide was determined in monkeys following a single injection and following four-hours continuous infusion<sup>56</sup>: the plasma half-life following a single injection is significantly shorter than that observed following continuous infusions; steady-state plasma concentration following continuous infusion, is achieved in 4–9 days; a higher concentration of oligonucleotide accumulates in the liver, kidney, heart, spleen and pancreas, than in other organs.

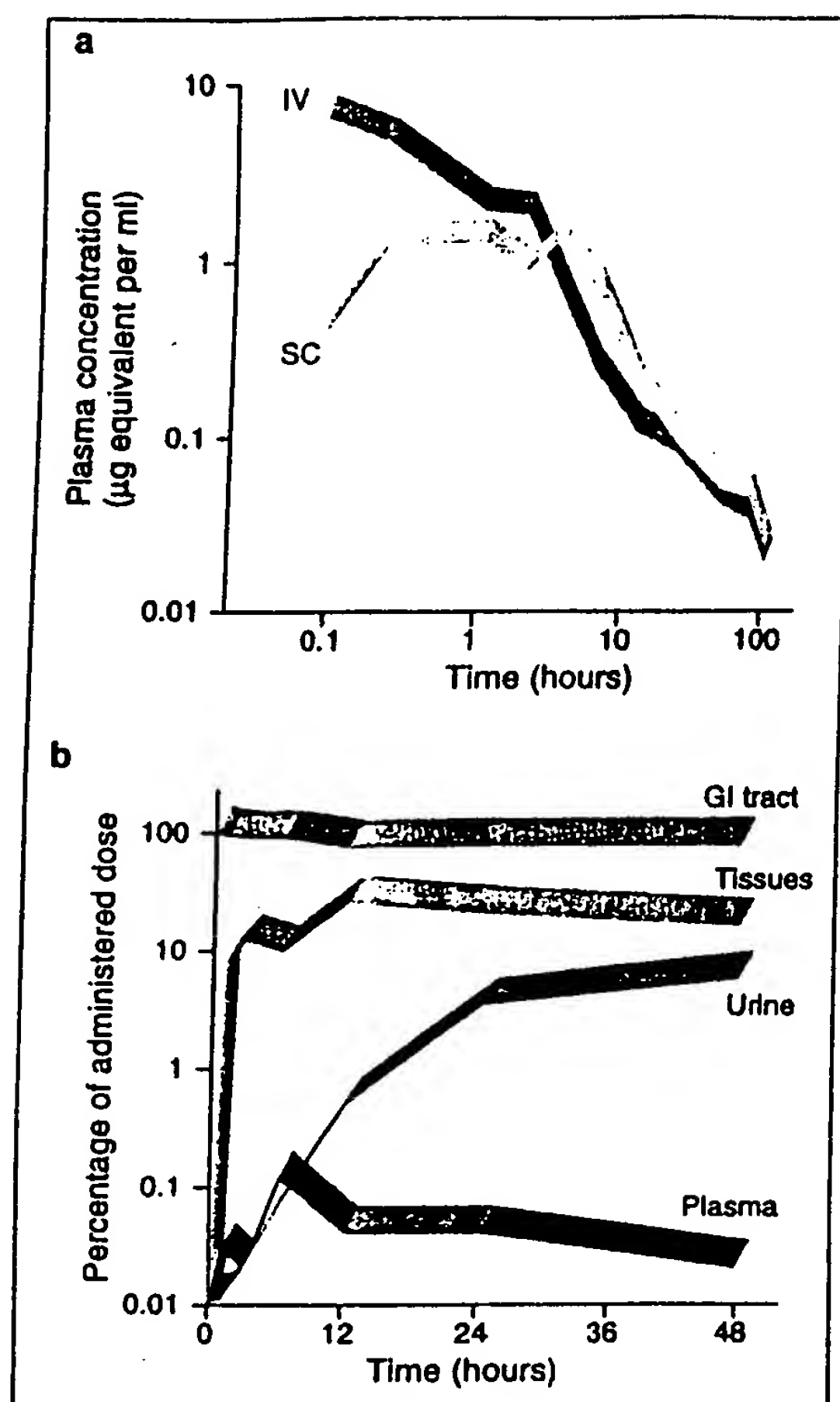


Figure 5

(a) Comparative plasma-clearance profile of GEM<sup>91</sup> following intravenous (IV) and subcutaneous (SC) administration (data reproduced from Ref. 55). (b) Absorption and disposition of hybrid oligonucleotide following oral gavage in rats; this figure shows the percentage of the dose (based on radioactivity level) in the gastrointestinal (GI) tract, the plasma, tissues, and being eliminated in the urine. (Further details of the studies are given in Ref. 73.)

In monkeys, the peak plasma concentration of PS-oligonucleotides correlates with concentration-dependent side-effects. We have shown that bolus intravenous

administration of PS-oligonucleotide in monkeys activates complement and prolongs partial thromboplastin time (aPTT), in a dose-dependent and infusion-rate dependent manner<sup>67</sup>. Slow intravenous infusion of the PS-oligonucleotide can prevent these side-effects from occurring, probably by lowering the peak plasma concentration<sup>67</sup>. Administration of PS-oligonucleotides by the subcutaneous, intramuscular, intraperitoneal, or intradermal routes may also help in reducing the side-effects caused by peak plasma concentration.

#### Pharmacokinetics of oligonucleotides in humans

Human clinical trials using PS-oligonucleotides delivered by intravenous infusion or intravitreal injection are currently being carried out (Table 1).

In one study, 17 patients with leukemia were given intravenous infusions of PS-oligonucleotide 20mer complementary to p53 mRNA, with doses ranging from 0.05 mg kg<sup>-1</sup> hr<sup>-1</sup> to 0.25 mg kg<sup>-1</sup> hr<sup>-1</sup> for 10 days<sup>68</sup>. The results show that the plasma concentration is linearly proportional to the dose administered, but elimination half-life is increased as a function of dose. Plasma concentration reaches a steady state in less than 24 hours, and up to 60% of the administered dose is cleared in urine.

We have been studying a PS-oligonucleotide 25mer (GEM<sup>91</sup>) complementary to the *gag* gene of HIV-1 (Refs 28,69). Administration of <sup>35</sup>S-labeled GEM<sup>91</sup> in six HIV-1 infected patients by two-hour intravenous infusion at a dose of 0.1 mg kg<sup>-1</sup> produced a peak plasma concentration of 295 ng ml<sup>-1</sup> by the end of the infusion period<sup>70</sup>. The plasma disappearance curve for GEM<sup>91</sup>-derived radioactivity can be described by the sum of the two exponentials, with half-life values of 0.18 and 26 hours. Analysis of the radioactivity in plasma by polyacrylamide gel electrophoresis (PAGE) indicates the presence of intact GEM<sup>91</sup> up to two hours following infusion.

Urinary excretion represents the major pathway of elimination of GEM<sup>91</sup>, with 49% and 70% of the administered dose being excreted in 24 hours and 96 hours, respectively, in the degraded form<sup>70</sup>. Escalating doses of GEM<sup>91</sup> (0.3, 0.5, 1 or 2.5 mg kg<sup>-1</sup>) in a two-hour infusion result in higher levels of GEM<sup>91</sup> in plasma<sup>71</sup>. The results of an open-label multiple-dose pharmacokinetic study in which GEM<sup>91</sup> was

Table 1. Phosphorothioate oligonucleotides currently in clinical trials

Oligo	Sequence (5'-3')	Target	Mode of delivery	Stage of clinical trials
ISIS 2922	GCGTTTGCTCTCTCTTCTTGCG	CMV retinitis	Intravitreal	Phase III
GEM <sup>91</sup>	CTCTCGCACCCATCTCTCTCCTTCT	HIV	Intravenous (iv)	Phase Ib/II
ISIS 2302	GCCCAAGCTGGCATCCGTCA	ICAM	iv	Phase II
ISIS 3521	GTTCTCGCTGGTGAGTTTCA	PKC	iv	Phase I
c-myb	TATGCTGTGCCGGGTCTTCCGGC	c-myb	iv	Phase I
ISIS 5132	TCCCGCCTGTGACATGCATT	c-raf	iv	Phase I

Abbreviations: CMV, cytomegalovirus; HIV, human immunodeficiency virus; ICAM, intercellular adhesion molecules; PKC, protein kinase C.



administered to patients every other day for 27 days (14 doses) by two-hour infusion at doses of 0.5, 1 or 2 mg kg<sup>-1</sup>, show that oligonucleotide does not accumulate in plasma following multiple dosing<sup>72</sup>.

#### Is oral administration of oligonucleotides possible?

As discussed previously, oligonucleotides administered by intravenous, intraperitoneal, subcutaneous, intradermal and intravitreal routes in animals show different bioavailability. We have also performed experiments to study the possibility of oral bioavailability of oligonucleotides. Initial studies were carried out with 25mer PS-oligonucleotides, which, when administered by oral gavage in rats, show very good stability in stomach contents for up to three hours, but are shown to have been degraded extensively in the small and large intestine contents. The degradation pattern suggests that the degradation is primarily by 3'-exonucleases. We have studied a hybrid oligonucleotide<sup>64</sup> (a PS-oligonucleotide carrying four 2'-O-methyl-ribonucleoside linkages at the 3' and 5' ends) for its stability in the gastrointestinal (GI) tract following oral gavage. It was notably more stable in the GI tract than is the PS-oligonucleotide<sup>73</sup>, and absorption of the hybrid from the GI tract into the body was time-dependent: approximately 25% of the oligonucleotide (based on radioactivity levels) was adsorbed in the 12 hours following oral gavage (Fig. 5b). Analysis of the absorbed radioactivity following extraction from various tissues and plasma indicated the presence of intact and degraded hybrid oligonucleotide<sup>73</sup>. Ongoing studies in mice of oral absorption of the hybrid oligonucleotide show a profile similar to that observed in rats. Furthermore, a chimeric oligonucleotide<sup>65</sup> containing PS- and methylphosphonate linkages shows better absorption than hybrid oligonucleotide, suggesting that a reduction in charges, or formulations that mask charges, may further increase the oral absorption. These preliminary experiments demonstrate that appropriately modified oligonucleotides can be delivered orally. The question of effective oral bioavailability remains to be answered, and further work in this direction is in progress.

In summary, pharmacokinetic studies demonstrate that: PS-oligonucleotides are absorbed after administration via various routes; they have a short plasma half-life; they distribute broadly to all perfused organs; and they are eliminated primarily in urine. In pharmacokinetic studies carried out using <sup>14</sup>C-labeled PS-oligonucleotides, more elimination of PS-oligonucleotide is observed in expired air than in urine<sup>53</sup>. Although oligonucleotides are rapidly cleared from plasma, they are retained in tissues for extended periods of time (Fig. 4). Oligonucleotides accumulate in, and are eliminated slowly from, the kidney, the liver and the bone marrow, suggesting that they could be administered less frequently if these are the disease target organs. However, side-effects might occur in these organs if oligonucleotides are administered frequently.

#### *In vivo* side-effects of oligonucleotides

The *in vivo* side-effects of PS-oligonucleotides may be the result of their polyanionic nature, length, base composition and sequence, or be due to their metabolites.

Studies carried out in mice and rats show that PS-oligonucleotides are well tolerated, having an LD<sub>50</sub> in excess of 500 mg kg<sup>-1</sup>. Dose-dependent side-effects observed in rats, following daily administration of PS-oligonucleotide, include splenomegaly, thrombocytopenia and elevation of the levels of the liver enzymes<sup>74</sup> aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Histopathological examinations have revealed multi-organ monocellular infiltrates, which are especially pronounced in kidney and liver, RE cell and lymphoid hyperplasia, and renal tubule degeneration/necrosis. The severity of these side-effects is dependent on the dose, and the frequency and duration of dosing. These side-effects may be sequence-dependent, but additional data are required to confirm that.

In monkeys, the toxicological profile of PS-oligonucleotides is very different from that observed in rats. Intravenous bolus administration of PS-oligonucleotide in monkeys causes a brief increase, followed by a prolonged decrease, in arterial blood pressure, and a transient decrease in peripheral total white blood cell and neutrophil counts<sup>67</sup>; complement activation and prolongation of aPTT have also been observed<sup>67</sup>. Side-effects are dose- and infusion-rate-dependent, and administering the same dose of PS-oligonucleotide by slow intravenous infusion produces a significant decrease in these side-effects<sup>67</sup>. In the same study, other PS-oligonucleotides of varying length and base composition were shown to produce similar side-effects, indicating that the side-effects are not sequence-dependent.

Side-effects caused by other PS-oligonucleotides have been reported in an independent study<sup>75</sup>. Activation of complement and prolongation of aPTT appear to be caused by phosphorothioate linkages of oligodeoxynucleotides, and may be due to their polyanionic nature; addition of protamine neutralizes both of these side-effects in *in vitro* studies<sup>76</sup>. Similarly, PS-oligonucleotides that have partially non-ionic backbones, RNA-modified segments, or secondary structures have reduced side-effects, suggesting that the side-effects are associated with the anionic nature of the phosphorothioate linkages of oligodeoxynucleotides<sup>77</sup>.

The safety profile of PS-oligonucleotides in rodents and monkeys is quite different. In monkeys, the side-effects seem to depend on the polyanionic nature of the oligonucleotide. Studies carried out with polyanionic molecular dextran sulfate in pigs<sup>78</sup> and rabbits<sup>79</sup> have indicated transient arterial hypotension, activation of complement, prolongation of aPTT and thrombocytopenia. These side-effects are also observed following infusion of dextran sulfate in humans<sup>80</sup>. In rodents, however, the side-effects may be caused by cytokine induction or by other factors, in

addition to the polyanionic nature of the oligonucleotide. Oligonucleotides containing the CG motif have been shown *in vitro* to induce cytokine production, including interleukin 12 (IL-12; Ref. 81). We have shown that a single dose of PS-oligonucleotide containing the appropriate nucleotide motifs induces cytokine expression, including IL-12, in mice<sup>82</sup>. Administration of IL-12 in mice causes thrombocytopenia, splenomegaly, elevation of serum transaminases, kupfer cell hyperplasia and mononuclear cell infiltration. In monkeys<sup>83</sup>, IL-12 administration causes thrombocytopenia, which is reversible<sup>84</sup>. It has not yet been established whether the safety profile of PS-oligonucleotides containing the CG motif is similar to that of PS-oligonucleotides without the CG motif.

These studies in mice, rats and monkeys show that the side-effects observed with PS-oligonucleotides may be caused, in part, by cytokine induction and the polyanionic nature of the oligonucleotide. As summarized in Fig. 6, complement activation and prolongation of aPTT result from high peak-plasma concentration, and can be avoided by slow infusion of PS-oligonucleotides. Other side-effects observed are dependent on dose, frequency and duration of administration. Many observed side-effects can be reduced through chemical modifications of oligonucleotide<sup>77</sup>.

#### Side-effects of oligonucleotides in humans

The results of animal studies demonstrate that PS-oligonucleotides show dose- and frequency of administration-dependent side-effects. Initial studies on humans are being carried out by administering the PS-oligonucleotide at very low doses. Intravenous infusion of 20mer PS-oligonucleotide for ten days at doses ranging from 0.05 mg kg hr<sup>-1</sup> to 0.25 mg kg hr<sup>-1</sup> causes no observable toxicity during infusion, or for four

weeks following the initiation of infusion. In one patient, however, infusion was discontinued because of an increase in serum AST and alkaline phosphatase. GEM<sup>®</sup>91 has been infused intravenously over a two-hour period in HIV-1-infected patients, with doses escalating from 0.1 mg kg<sup>-1</sup> to 2 mg kg<sup>-1</sup>. Analysis of blood from patients given GEM<sup>®</sup>91 at 2 mg kg<sup>-1</sup> shows an increase in aPTT (Ref. 85). The effect on aPTT is highest at the end of the infusion, where aPTT is prolonged by 36%. Normalization of the aPTT occurred in parallel with GEM<sup>®</sup>91 clearance; four hours after the cessation of GEM<sup>®</sup>91 infusion, aPTT values are normal. The prolongation of aPTT is not associated with the clotting factor deficiency: the plasma levels of the clotting factors involved in the endogenous pathway, including the contact phase (factors VIII, IX, XI, XII, HMW and kininogen), as well as those of the common pathway (factors V, X, and II), and fibrinogen, remain unchanged throughout the study. This increase in aPTT is not specific to GEM<sup>®</sup>91, but is observed with administration of PS-oligonucleotides in general<sup>66,77,86</sup>.

The safety profile of PS-oligonucleotides administered intravitreally at doses of 83, 165, 330 or 495 µg to 22 AIDS patients with refractory cytomegalovirus (CMV) retinitis (28 eyes), has been studied. The most frequently observed ocular side-effects are anterior and posterior chamber inflammation. Marked retinal toxicity is observed only at the highest dose in the only patient to receive 495 µg. In this study, PS-oligonucleotide administration was by bi-weekly dosing<sup>87</sup>.

#### Conclusions and future directions

It is clear from some of the studies mentioned in this review and many other published reports that PS-oligonucleotides show more sequence-specific antisense activity in animal models than in cell culture experiments. Perhaps the mechanism of cellular uptake, localization, interaction with non-targeted genes, accessibility to the gene, metabolism and clearance of metabolites is different in animals than in cell culture models. If so, which is the more appropriate model for studying antisense oligonucleotides: cell culture or animal models?

The activity of an oligonucleotide in tissue culture depends on many variables, including cell type, cellular uptake, percentage of serum in the cell culture medium, duration of the experiment, end point, concentration of oligonucleotide, degradation of oligonucleotide and the impact of its metabolites, and the use of lipid carriers or other delivery devices, including microinjection. The effects of oligonucleotides in cell culture may be affected by their polyanionic nature and 'suspicious' nucleotide domains (e.g. CG) of oligonucleotide sequences. The activity of an oligonucleotide in animal models depends on dose, route of administration, disposition in tissues, *in vivo* stability, and half-life of the oligonucleotide. In animal models, side-effects caused by the immunogenic or polyanionic nature of the oligonucleotide may interfere in assessing the antisense activity, and may vary for animals

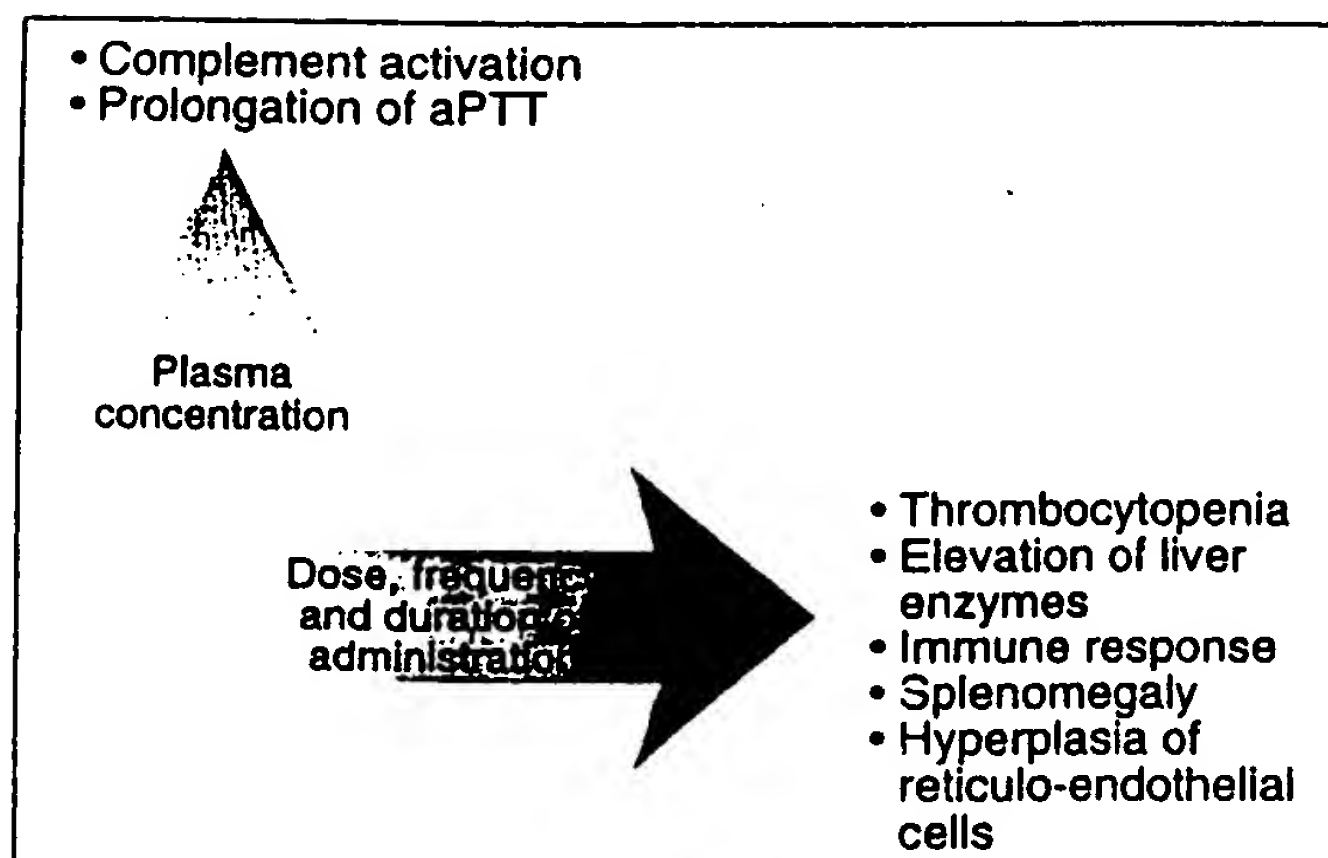


Figure 6

Two types of side-effects have been observed in animals. In monkeys, side-effects observed include prolongation of partial thromboplastin time (aPTT) and complement activation. These are directly correlated to peak plasma concentration. In rats and monkeys, certain side-effects (e.g. thrombocytopenia, elevation of transaminases and splenomegaly) are related to the dose, and the frequency and duration of dosing.

(including nude, SCID, or normal mice). The results of studies in cell culture suggest that animals may be more appropriate models for confirming the antisense effects of oligonucleotides, following tissue culture screening.

Pharmacokinetic studies of PS-oligonucleotides in animals suggest that much of the administered dose of an oligonucleotide and its metabolites (based on radioactivity levels) are retained in the tissues. These

metabolites include shorter fragments of administered oligonucleotides generated primarily by 3' exonuclease digestion<sup>50,88</sup>; however, at later time points we have observed metabolites generated by 5' exonuclease digestion, or by both 3' and 5' exonuclease digestion (J. Temsamani and S. Agrawal, unpublished data), including nucleotides (monothiophosphate or monophosphate) and nucleosides that are present in both the intracellular and extracellular compartments.

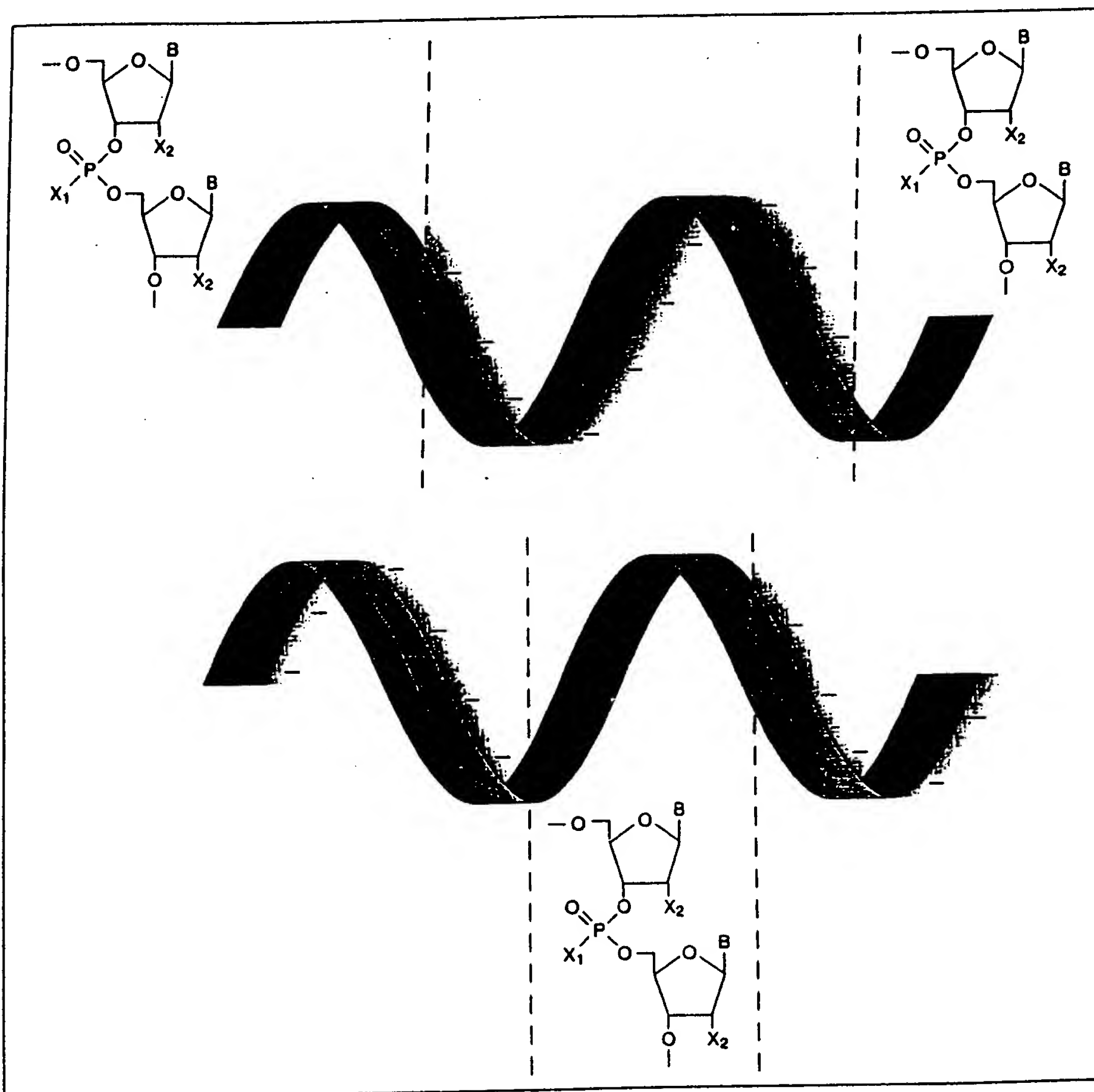


Figure 7

Mixed backbone oligonucleotides (MBOs) provide a handle on modulating biological, pharmacodynamic and pharmacokinetic profiles. The advantage of MBOs is that, while they retain the advantage of PS-oligonucleotide (RNase H), the side-effects inherent in PS-oligonucleotide can be minimized. The positioning of the modified oligodeoxynucleotide or oligoribonucleotide in a PS-oligonucleotide is critical for its desired properties. For example, placement of methylphosphonate linkages ( $X_1 = \text{CH}_3$ ,  $X_2 = \text{H}$ ) at the ends reduces the overall polyanionic-related side-effects, and increases the *in vivo* stability by protecting both ends of the PS-oligonucleotide from degradation. Similarly, placement of 2'-O-methylribose nucleotide ( $X_1 = \text{S}$ ,  $X_2 = \text{OCH}_3$ ) provides increased affinity and *in vivo* stability. More stable oligonucleotides have two advantages: longer duration of action will mean less frequency of dosing, and the presence of fewer degradation metabolites will decrease the potential for unwanted side-effects from such metabolites. Placing the modified segment of oligodeoxynucleotide or oligoribonucleotide in the center of the PS-oligonucleotide provides a further handle on modulating the rate of degradation, on the nature of metabolites being generated *in vivo*, and on the elimination of the metabolites.



The fate of these metabolites is unknown. The safety profiles of various PS-oligonucleotides studied are quite similar, but further studies are required to establish which structural features of PS-oligonucleotides are responsible for which side-effects. At least six PS-oligonucleotides are currently being tested in human clinical trials (Table 1); for example, ISIS 2922 is in Phase III clinical trials, and it is claimed to be effective in controlling CMV replication following intravitreal injections<sup>87</sup>. GEM<sup>®</sup>91 is the PS-oligonucleotide that has been studied most extensively following intravenous administration, and it is currently in Phase II clinical trials. Other oligonucleotides are at various stages in their clinical development.

While PS-oligonucleotides are promising as the first generation of oligonucleotides, efforts are being made to further improve their biological potency and safety profile. Some of the properties that must be improved in the next generation of antisense oligonucleotides are listed in Box 1. To this end, we have designed mixed backbone oligonucleotides (MBOs) as second-generation oligonucleotides (Fig. 7); these contain segments of phosphorothioate oligodeoxynucleotides and appropriately placed segments of modified oligodeoxy- or oligoribonucleotides<sup>16-26,89,90</sup>. MBOs have segments of phosphorothioate linkages (providing an RNase H substrate), and other segments of other modified oligonucleotides, such as methylphosphonate, which is non-ionic, and very resistant to nucleases or 2'-O-alkyloligoribonucleotides, which have higher affinity and improved nuclease stability. MBOs are more potent than their PS-oligonucleotide counterparts in cell culture experiments, and they show tissue disposition profiles similar to those of PS-oligonucleotides, but with a significant increase in *in vivo* stability, which may enable less frequent administration<sup>64,65</sup>. These MBOs have fewer, less severe side-effects related to their polyanionic nature<sup>64</sup> and immunogenic response (Fig. 7; Ref. 91).

In MBOs, the choice of oligonucleotide modification and its placement in the oligonucleotides is crucial. We have recently studied MBOs in which the modified DNA or RNA segment is placed in the center (Fig. 7). While centrally modified oligonucleotides<sup>92</sup> (e.g. containing methylphosphonate linkages) retain their biological potency *in vitro* and *in vivo*<sup>93</sup>, they show significantly fewer side-effects in rats than do PS-oligonucleotides. Also, polyanionic-related side-effects are significantly reduced, because the number of contiguous phosphorothioate linkages has been reduced in these MBOs (S. Agrawal and D. Shaw, unpublished data).

While MBOs are being pursued as the second generation of antisense oligonucleotides, a number of newer analogs of DNA (Ref. 26) will provide further tools for modulating the properties of antisense oligonucleotides.

Many questions are being raised about the promise of the antisense therapeutic approach based on cell culture studies, and animal studies are providing some clues and answers. Preliminary evidence of clinical

efficacy against CMV has been claimed with antisense oligonucleotides, and ongoing clinical trials with antisense oligonucleotides will further expand the knowledge of the safety and efficacy profiles of first-generation antisense oligonucleotides. The knowledge gained from the first generation of oligonucleotides will lead to the development of second-generation oligonucleotides, which should provide improved safety and efficacy.

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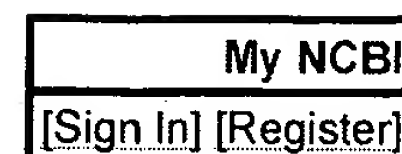
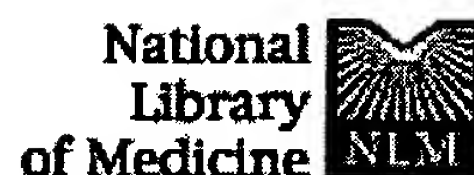
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## Targeting of antisense DNA: comparison of activity of anti-rabbit beta-globin oligodeoxyribonucleoside phosphorothioates with computer predictions of mRNA folding.

Jaroszewski JW, Syi JL, Ghosh M, Ghosh K, Cohen JS.

Department of Organic Chemistry, Royal Danish School of Pharmacy, Copenhagen.

To assess the usefulness of computer-assisted modeling of mRNA as an aid in design of antisense DNA, the efficiency of inhibition of translation of rabbit beta-globin mRNA by various antisense sequences was compared with calculated structures of the mRNA. The model obtained by consideration of 30 lowest-energy computer-simulated structures is consistent with the high accessibility of the AUG initiation codon region known from digestion with nucleases and with previous antisense inhibition studies reported in the literature. Additional antisense inhibition data were obtained with 20-mer phosphorothioate oligonucleotides, targeted to regions of beta-globin mRNA differing moderately in their degree of participation in intramolecular folding. The efficiency of translation arrest by the oligonucleotides in cell-free expression systems (wheat germ extract and rabbit reticulocyte lysate) was obtained by measuring incorporation of [35S]methionine into total protein, and corrected for sequence-nonspecific inhibition using brome mosaic virus mRNA. In the presence of RNase H (wheat germ system), the inhibitory activity of the oligonucleotides showed correlation with the calculated secondary structure of mRNA, in particular at low oligonucleotide-to-mRNA ratios (correlation coefficient, 0.95). No correlation was observed in the reticulocyte lysate system, in which the inhibition is mediated by translational arrest.

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the folding of the protease sequences when added as separate molecules, both *in vitro* and *in vivo*<sup>15</sup>. One way in which cells change the quantitative properties of proteins is to make allosteric effectors; this method is reversible and requires the continual presence of the effector. Perhaps another method useful in say, terminal differentiation, is the production of separate sterile chaperones that irreversibly change the properties of certain specific proteins by influencing their folding.

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## A good antisense molecule is hard to find

Andrea D. Branch

Antisense molecules and ribozymes capture the imagination with their promise of rational drug design and exquisite specificity. However, they are far more difficult to produce than was originally anticipated, and their ability to eliminate the function of a single gene has never been proven. Furthermore, a wide variety of unexpected non-antisense effects have come to light. Although some of these side effects will almost certainly have clinical value, they make it hard to produce drugs that act primarily through true antisense mechanisms and complicate the use of antisense compounds as research reagents. To minimize unwanted non-antisense effects, investigators are searching for antisense compounds and ribozymes whose target sites are particularly vulnerable to attack. This is a challenging quest.

**ANTISENSE STRATEGIES LOOK** almost too easy on paper. Simple and elegant schemes can be drawn for both antisense oligodeoxynucleotides (ODNs - short DNA molecules intended to bind to and inhibit target RNAs through complementary Watson-Crick base pairing) and bioengineered ribozymes (catalytic RNA molecules intended to bind and cleave target RNAs). Scientists seek to use these molecules to ablate selected genes and thereby understand their functions, and

pharmaceutical developers are working to find nucleic acid-based therapies. However, the antisense field has been turned on its head by the discovery of 'non-antisense' effects, which occur when a nucleic acid drug acts on some molecule other than its intended target - often through an entirely unexpected mechanism. Non-antisense effects are not necessarily bad. Indeed, some may prove to be a boon to the pharmaceutical industry because they offer an added source of potency. However, their unpredictability confounds research applications of nucleic acid reagents.

Non-antisense effects are not the only impediments to rational antisense drug

design. The internal structures of target RNAs and their associations with cellular proteins create physical barriers, which render most potential binding sites inaccessible to antisense molecules. For Watson-Crick base pairing to occur, nucleic acid drugs must be complementary to exposed regions in their target RNAs and must co-localize with them. When these requirements are met, true antisense effects are enhanced, and unwanted non-antisense effects are minimized. However, optimization is a time-consuming process. Currently, effective nucleic acid drugs must be selected from large pools of candidates. Streamlined approaches for (irrational) *in vivo* selection are needed to speed the discovery of active molecules.

**Non-antisense effects: quicksand for some, diamond mines for others**

The potential of nucleic acid drugs to deliver 'exquisite specificity'<sup>1</sup> is frequently cited: antisense methods are credited with offering 'the specificity of the genetic code and the versatility of targeting any number of proteins'<sup>2</sup>; and it is said that a therapeutic ribozyme 'can be designed to interact only with its target, and the target is expected to appear only once in the genome, giving one a high degree of assurance that the target - and only that target - has been inhibited'<sup>3</sup>. However, it has never been proven that antisense drugs have the capacity to knock out just one gene, although both ODNs and bioengineered ribozymes can undeniably hit their intended targets<sup>4,5</sup>. The powerful appeal of antisense strategies has been a mixed blessing. The twin concepts that effective antisense reagents are easy to

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design and that they selectively home in on their targets have overshadowed the cautionary messages in articles such as 'Antisense has growing pains'<sup>6</sup>, 'Can hammerhead ribozymes be efficient tools to inactivate gene function?'<sup>7</sup>, and 'Does antisense exist? (It may, but only under very special circumstances)'<sup>8</sup>.

The purpose of this article is to review the factors that make and break specificity in antisense applications and to discuss the need to judge therapeutic compounds and research reagents by separate standards. Only antisense molecules<sup>9-11</sup> and ribozymes<sup>12,13</sup> designed to inhibit RNA targets are considered here, but many of the principles apply to other nucleic acid drugs, such as those used to correct DNA mutations<sup>14</sup>, to alter RNA splicing<sup>15</sup>, and to control gene expression by forming triple helices with DNA (Ref. 16).

Non-antisense effects pose a dilemma for the pharmaceutical industry<sup>17</sup>. These effects include the stimulation of B-cell proliferation<sup>18</sup> and the inhibition of viral entry into cells<sup>19</sup>, responses which are potentially useful. Non-antisense ODNs are already being developed as adjuvants to boost the efficacy of immunotherapies and vaccines<sup>20</sup>. Phase III clinical trials of ISIS 2922 (Ref. 21), a phosphorothioate oligonucleotide (S-ODN) that induces both antisense and non-antisense effects, are also under way in patients with cytomegalovirus-associated retinitis<sup>22</sup>. It is hoped that this compound's diverse mechanisms of action will yield a single drug that provides many of the benefits of combination therapy. However, as Anderson and colleagues have observed, characteristics that are advantages in pharmaceutical drugs can be disadvantages in research reagents<sup>21</sup>. Thus, a safe and effective nucleic acid drug that slows the progression of AIDS would be of tremendous value, even if it were to act by inhibiting a perplexing combination of viral proteins rather than by binding to HIV RNA as originally intended. However, this same compound would be useless as an agent to selectively destroy HIV RNA, and could be ruinous if used in experiments of HIV molecular biology without knowledge of its mechanism of action. Because a single, well-understood mechanism of action cannot be assumed, non-antisense effects create major difficulties for gene hunters. Years of investigation can be required to figure out what an 'antisense' molecule is actually doing, as discussed further below.

Non-antisense effects also have a downside for pharmaceutical developers.

Because knowledge of their underlying mechanisms is typically lacking, non-antisense effects muddy the waters. They make true antisense drugs more difficult to design and harder to commercialize. Furthermore, they can be a source of toxicity.

#### **All drugs are dirty: clinical benefit is the pharmaceutical gold standard**

Stanley Crooke (Isis Pharmaceuticals) stresses that 'a vast body of experience says that no drug is entirely selective'<sup>23</sup>. Because biologically active compounds generally have a variety of effects, dose-response curves are always needed to establish a compound's primary pharmacological identity. Antisense compounds are no exception. As is true of all pharmaceuticals, the value of a potential antisense drug can only be judged after its intended clinical use is known, and quantitative information about its dose-response curves and therapeutic index is available.

It may be surprising to hear antisense molecules described in the same terms as conventional drugs, but, in fact, nucleic acid drugs should not be thought of as magic bullets. Their therapeutic use will require vigilant monitoring. Compared to the dose-response curves of conventional drugs, which typically span two to three orders of magnitude, those of antisense drugs extend only across a narrow concentration range. Both *in vitro* and *in vivo*, less than a factor of ten often separates the concentration producing no antisense effect from that producing the full antisense effect<sup>24</sup>. Steep dose-response curves commonly indicate that a drug has multiple, synergistic mechanisms of action<sup>24</sup>. A drug with a narrow therapeutic window can be potent and extremely valuable, but can also be tricky to use safely. Since the ratio of antisense to non-antisense effects drops sharply outside a restricted concentration range, it will be challenging to obtain consistent therapeutic results.

#### **Mother Nature's cruel antisense jokes lead to tougher experimental standards**

Their powerful allure and favorable press have often caused the problems associated with antisense reagents to be trivialized. In some cases, relaxed standards have been applied. Arthur Krieg (University of Iowa) provided insight into the need for stricter quality control when he shared the results of an informal poll. He reported that 'the estimate that many people have given me of the percentage of accurate published antisense papers

ranges from 50% of them being accurate to 5% being accurate'<sup>22</sup>.

As discussed previously, when an antisense molecule causes a biological effect, it can be extremely difficult to determine whether the change occurred because the reagent interacted specifically with its target RNA, or because some non-antisense reaction - involving other nucleic acids or proteins - was set in motion<sup>8,25</sup>. When attempting to distinguish between antisense and non-antisense effects, a common strategy has been to use an oligonucleotide in which the sequence of the antisense oligonucleotide is altered. Unfortunately, not all non-antisense effects can be readily detected by this approach, as illustrated by studies of antisense therapies for chronic myeloid leukemia. In this disease, a chromosomal translocation often produces the Philadelphia chromosome, resulting in the synthesis of an oncogenic fusion protein, BCR/ABL. The mRNA for this protein has been regarded as an ideal target for antisense therapies. Several groups have reported inhibition of leukemic cell proliferation by anti-BCR/ABL antisense oligonucleotides. In fact, Vaerman and co-workers cite 16 publications reporting promising findings<sup>26</sup>. However, they discovered that a disappointing, non-antisense mechanism was responsible for their own results, adding weight to studies showing that S-ODNs block proliferation through non-antisense mechanisms (reviewed in Ref. 26). Recent work indicates that cytotoxic ODN breakdown products are responsible for the antiproliferative effects observed<sup>27</sup>. These studies strongly underscore the need to test numerous control ODNs when carrying out antisense research, and to maintain a high index of suspicion.

C. A. Stein (Columbia University) has reviewed many 'non-sequence-specific' (non-antisense) effects caused by S-ODNs, providing dramatic examples of the havoc that has resulted when S-ODNs have unleashed their surfeit of cryptic information. S-ODNs are used because their modified backbones confer nuclease resistance. However, they bind avidly to many proteins, forming complexes with dissociation constants one to three orders of magnitude lower than those of phosphodiester ODNs. In a test of B-cell proliferation and differentiation, S-ODNs were two logs more potent than phosphodiester ODNs of the same sequence<sup>28</sup>. According to Stein, S-ODNs have 'bamboozled' many researchers by inducing biological effects that mimic, and are mistaken for, true and desired antisense effects<sup>8,19</sup>.



Addressing the manifest need for stricter experimental standards, Arthur Krieg and C. A. Stein (editors of the journal *Antisense and Nucleic Acid Drug Development*) have published guidelines for designing antisense studies<sup>1</sup>. Recently, the need to use pure oligonucleotide reagents has been stressed. The selective publication of expected (positive) results is being actively discouraged. The confusion that has thus far occurred indicates that each new 'antisense' molecule needs to be tested exhaustively.

#### How close do current antisense techniques come to single-gene accuracy?

While the ability to knock out a single gene is a luxury in a pharmaceutical compound, specificity is a key feature of a reagent to be used in a research setting. Although single-gene accuracy is not essential for an experimental reagent to be useful, the extraneous perturbations it causes need to be identified. Additionally, as alternative approaches for selective gene ablation (such as the production of genetic knockouts) improve and become easier to carry out, it will be important to know how antisense techniques compare in terms of time, expense and selectivity. This comparison awaits additional information about antisense specificity.

Unfortunately, quantitative data about the magnitude of antisense-induced side reactions are limited. Most of the information is extrapolated from experiments in which the impact of an antisense compound is measured on only a small number of molecules: the intended target RNA, a housekeeping gene, and perhaps a few control RNAs. An antisense molecule is typically taken to be 'specific' if two criteria are met: (1) there is no gross loss of cell viability, and (2) the levels of the target RNA and its associated protein fall much more than those of the control RNAs. However, this type of experimental design is too limited in scope to provide information about global changes in the RNA and protein populations. It does not provide even a rough measure of the signal-to-total noise ratio. Unlike the analysis of Scatchard plots, which allows the interactions between a ligand and a complex mixture of proteins to be explored, this design looks at three or four RNAs and projects the impact on the remaining  $10^5$  genes. As an additional shortcoming, it provides no direct information about interactions between the antisense molecule and proteins, even though these interactions may lead to the major effects caused by 'antisense' molecules. Because it could provide a before-and-after

snap-shot of the protein population, high-resolution two-dimensional gel electrophoresis<sup>23</sup> might shed light on the spectrum of changes induced by antisense molecules. However, a recent round-table discussion suggested that there are no published studies in which this technique has been utilized to evaluate antisense specificity<sup>22</sup>.

So far, the concept that an antisense molecule can selectively knock out a single gene appears to have been untested. In the future, several techniques, in addition to two-dimensional gel electrophoresis, might be employed to investigate antisense specificity. For example, as the repository of sequenced genes grows, it will be possible to identify RNAs that contain regions complementary to an antisense molecule and to measure the impact of antisense treatments on these bystander molecules. In addition, broad surveys of mRNA populations could be conducted. To identify changes induced by antisense treatments, RNA from treated and control cells could be reverse-transcribed and the resulting cDNA populations analyzed either by differential display, which separates cDNAs electrophoretically, or by hybridization to gene chips, which are being developed to allow the quantitative monitoring of gene expression patterns<sup>30</sup>. Should unanticipated changes be detected by such surveys, other techniques could be used to distinguish those caused by lack of specificity from those reflecting downstream consequences of the intended antisense reaction. Information about the number of accidental hits and about the nature of the interactions responsible for the changes in the expression of other genes would be useful and would guide future drug development. Today's peak specificity, whatever it is, will almost certainly rise as current strategies are optimized and advances in nucleic acid chemistry bring derivatives with fewer side effects. New compounds are currently under investigation<sup>17,31</sup> and additional derivatives can be expected in the future.

#### Theoretical limits of specificity

Theoretical calculations provide a useful perspective on antisense specificity. The haploid human genome contains about  $3 \times 10^9$  bases. In a random sequence of this size, any sequence that is 17 nucleotides long or longer would have a high probability of occurring only once – of being unique. To knock out a single gene, an intervention would have to distinguish a 17-base perfect match from one with a single-base mismatch.

In considering whether ODNs have the requisite power of discrimination, it is crucial to know their mechanism(s) of action. These mechanisms may differ from cell type to cell type and may depend upon the exact nature of the target RNA and the ODN. However, there is strong evidence that in several systems, including *Xenopus* oocytes<sup>32</sup> and permeabilized cells<sup>33</sup>, the target RNA is destroyed by the action of RNase H. RNase H activities cleave the RNA component of DNA–RNA hybrids. They do not require long hybrid regions as substrates. In fact, *in vitro*, RNase H can cleave a hybrid containing only 4 bp (Ref. 34). In *Xenopus* oocytes, as few as 10 bp are sufficient<sup>35</sup>. For standard ODNs, it is likely that 10 bp are also sufficient in human cells; in the case of certain chemically modified nucleotides, it is proven that as few as 7 bp can lead to cleavage<sup>36</sup>. Random sequences the length of the human genome contain an average of 3000 copies of each 10-nucleotide sequence (10-mer). Thus, it is extremely likely that any particular 10-mer will occur in many RNAs. When an ODN complementary to this 10-mer is introduced into a cell, all of the RNAs containing this 10-mer are at risk for RNase H-mediated cleavage. Of course, not all 3000 copies will be susceptible to cleavage: many will not be present in transcripts, and many that are present in transcripts will be inaccessible. However, if even 1% of the 3000 are hit, 30 genes will be directly affected. Furthermore, the number of 'at risk' sites is probably more than an order of magnitude greater than 3000 for two reasons: (1) ODNs typically contain 20 or more bases, each 20-mer contains 11 10-mers, and each 10-mer would be present 3000 times, on average; and (2) in all likelihood, RNase H does not require 10 consecutive bp for cleavage. Because RNase H requires only a short hybrid region, it is not possible to increase specificity by increasing the length of the ODN. In fact, increasing the length beyond the minimum is likely to have the opposite effect, by stabilizing binding to mismatched sequences, as illustrated in Fig. 1.

Based on studies performed in *Xenopus* oocytes, Woolf and co-workers concluded that it is probably not possible to obtain cleavage of an intended target RNA without also causing at least partial destruction of many non-targeted RNAs (Ref. 35). The ratio of intended to unintended hits will depend on a complex and unpredictable combination of factors that determine whether the antisense molecule and the potential targets co-localize and

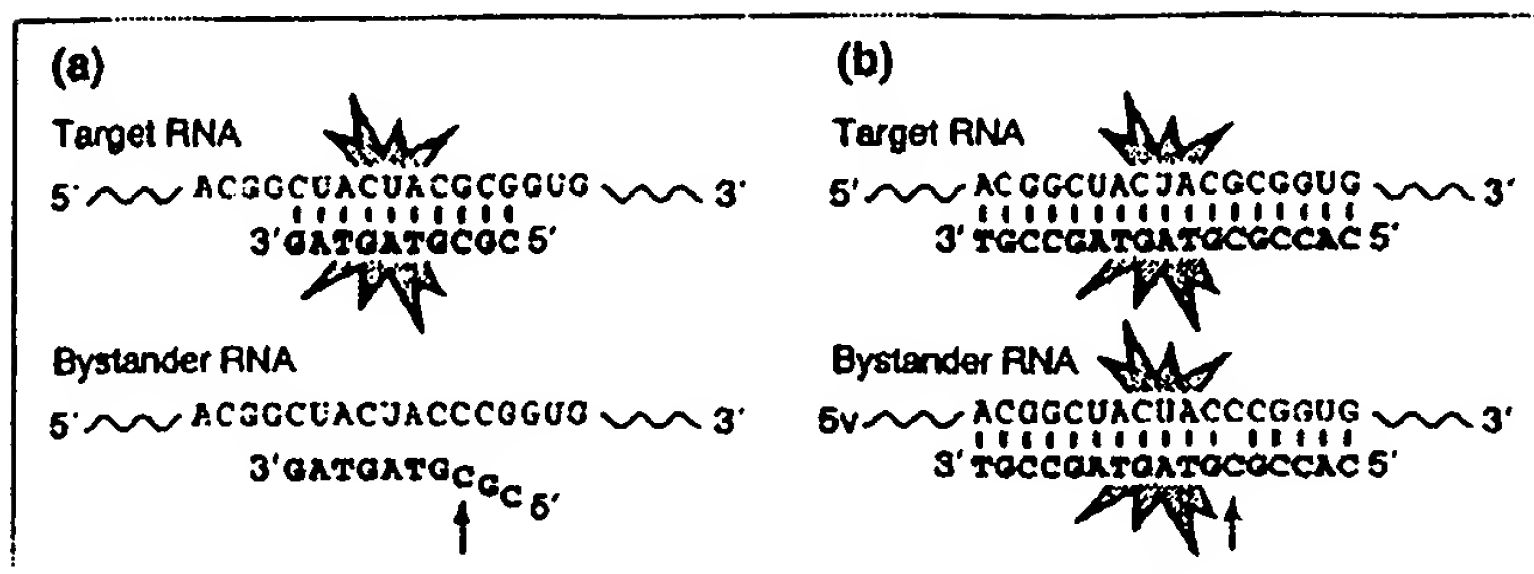


Figure 1

More is not always better. (a) A relatively short antisense ODN causes destruction of its intended target RNA but not a bystander RNA. This discrimination is possible because the ODN does not form enough base pairs with the bystander RNA to promote stable binding and RNase H-mediated cleavage. (b) A longer ODN annihilates both the target and the bystander, indiscriminately. From the standpoint of the gene hunter, an unfortunate situation exists. In general, an ODN short enough to discriminate between an RNA containing a perfect match and an RNA containing a one-base mismatch is so short that its perfect complement occurs in many different RNAs in a human cell. Thus, although it can distinguish between perfect and imperfect matches, the ODN cannot selectively destroy its target RNA. To overcome this problem, the second generation of ODNs will need special design features to enhance their specificity. In the diagrams, the 'explosion' denotes RNA cleavage by RNase H, ODNs are presented in boldface type, and sequences complementary to all or part of the ODN appear in regular lettering with the remainders of the target and bystander RNAs depicted by wavy lines; black arrows identify a nucleotide mismatch between the bystander RNA and the ODN (the bystander and the target RNA differ at this position).

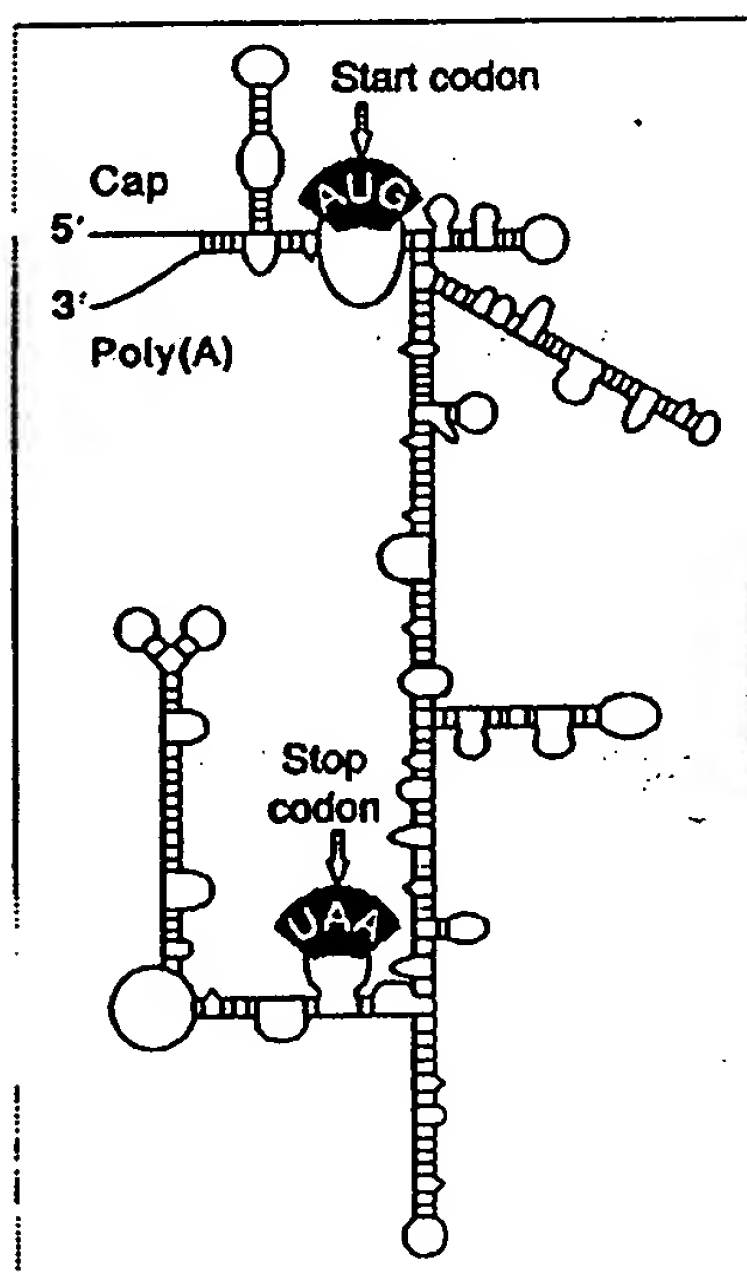


Figure 2

As illustrated by this secondary structure map of mouse  $\beta$ -globin mRNA, RNA molecules have an intricate array of intramolecular Watson-Crick bonds, which greatly diminish the portion of the molecule available for binding to antisense compounds and ribozymes. The positions of base pairs were determined by treating globin mRNA with structure-sensitive nucleases *in vitro*. Redrawn from Ref. 40, with kind permission.

whether the complementary sites in the RNAs are buried under proteins or are involved in intramolecular bonds that make them inaccessible. In the future, even as improvements in antisense chemistry reduce oligonucleotide binding to proteins, the specificity limits imposed by RNase H will remain and will be important to keep in mind when evaluating antisense strategies.

Target site recognition by bioengineered ribozymes is determined by Watson-Crick base pairing and thus has limits of specificity similar to those of ODNs. Ribozymes bind to their target RNAs through a recognition sequence of variable length. Somewhat counter-intuitively, a ribozyme with the potential to form a larger number of base pairs with its target RNA does not necessarily have a greater power to discriminate between its intended target and a related bystander RNA than a ribozyme with a shorter recognition sequence. In fact, extending the length of the recognition sequence may reduce a ribozyme's ability to discriminate<sup>37</sup>. It remains to be determined whether there are recognition sequence lengths that are both short enough to allow RNAs that differ from the target at a single nucleotide to be spared cleavage and long enough to allow a unique RNA to be selectively destroyed<sup>38</sup>. It will not be surprising if bioengineered ribozymes are incapable of knocking out single genes, as contemplated by Bertrand

and co-workers<sup>7</sup>. Most of these molecules are derived from either hammerhead or hairpin ribozymes<sup>13</sup>. In their natural setting, these ribozymes are covalently attached to their cleavage sites. They self-cleave precursor molecules of subviral (viroid) pathogens<sup>39</sup>. To fulfill their duties, these ribozymes have only to select their target site from the limited number of choices available in the same (small) RNA molecule. Thus, in terms of specificity, bioengineered ribozymes are expected to outperform their natural counterparts. Of course, besides binding to unintended RNAs through Watson-Crick and/or non-Watson-Crick interactions, ribozymes, like other RNAs, are highly charged molecules and have the potential to bind to cellular proteins, thereby producing biologically significant (non-antisense) effects.

As regards the theoretical limits of antisense specificity, it is important to remember that the genome is not a 'random sequence'. Sequences that constitute 'good' antisense targets in one RNA may occur in other RNAs at a higher or lower frequency than random chance would predict. One anecdote reveals how the redundancy of biological sequences could plague antisense methods. A conserved 350-base region at the 5' end of the hepatitis C virus is considered to be a potential target for antisense drugs. This short region contains a particular 10-mer that is also present in 62 known human mRNAs (Ref. 25), and it contains two 17-mers that occur in known human DNA sequences. Ultimately, the tendency for biological sequences to be reused may limit the specificity of strategies that rely solely on Watson-Crick base pairing for recognition. This tendency will become amenable to detailed analysis soon, as more complete data about human gene sequences become available.

#### The three As of antisense-mediated gene ablation: access, access and access

Inside cells, it is obviously not possible to improve specificity by raising the temperature or changing the ionic strength, manipulations that are commonly used to reduce background binding in nucleic acid hybridization experiments *in vitro*. Thus, alternative strategies are needed to enhance specificity within cells. One approach has been to deploy multiple antisense compounds, each directed against a different site in the same target RNA and thereby achieve annihilation by molecular triangulation. In addition, successful efforts have been made to exploit the fact that not all



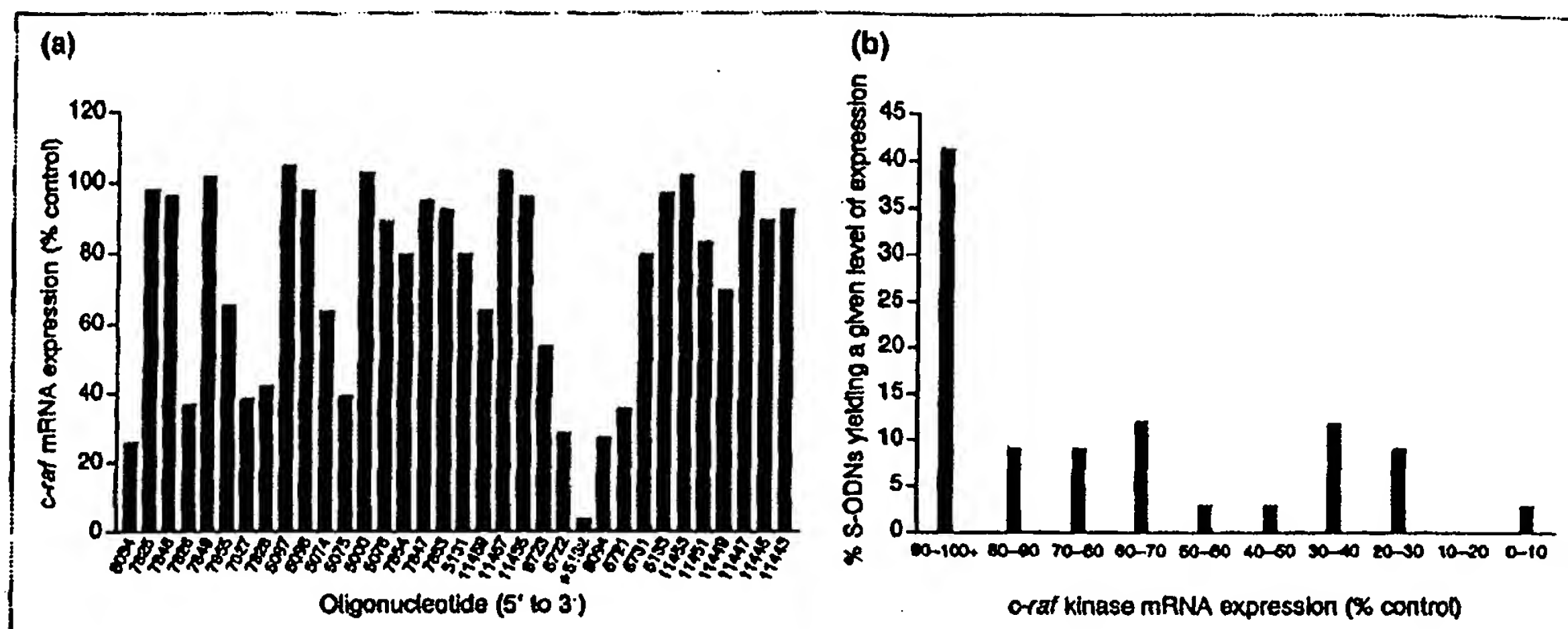


Figure 3

Superior S-ODNs can be found, but they are in the distinct minority. (a) Northern hybridization analysis revealed that, of 34 S-ODNs applied to A549 lung carcinoma cells, only one (5132, marked by an asterisk) caused a greater than fivefold reduction in the level of the target, *c-ras* kinase mRNA. Redrawn from Ref. 42, with kind permission. (b) Treatment with the majority of the S-ODNs had minimal effect and resulted in levels of the target mRNA that were 50% or more of the level in control cells.

portions of an RNA molecule are equally exposed. If a 10-mer complementary to an antisense ODN occurs in an accessible site in a target RNA and in a protected portion of a bystander, the target will be preferentially destroyed. The challenge is to identify antisense molecules that are complementary to vulnerable sites in target RNAs. This is hard to do. RNAs are complex molecules with intricate internal structures<sup>40</sup>, as illustrated by the diagram of  $\beta$ -globin mRNA (Fig. 2).

Recent studies emphasize the extent to which native RNA structure restricts the binding of ODNs. Milner and co-workers<sup>41</sup> tested the ability of 1938 ODNs (ranging in length from monomers to 17-mers) to bind to a 122-nucleotide RNA representing the 5' end of  $\beta$ -globin mRNA. They found that 'surprisingly few' ODNs bound stably to the mRNA, and concluded that binding is probably 'confined to those regions in the RNA which provide an accessible substructure'<sup>41</sup>. Using short (7 and 8 nucleotides) antisense molecules modified with C-5 propyne pyrimidine and phosphorothioate internucleotide linkages, Wagner and co-workers<sup>36</sup> also determined that the structure of the target RNA is a 'major determinant of specificity'.

Because it is very difficult to predict what portions of an RNA molecule will be accessible *in vivo*, effective antisense molecules must be found empirically by screening a large number of candidates for their ability to act inside cells. Monia and co-workers used northern hybridization to screen 34 20-nt long

S-ODNs complementary to *c-ras* kinase and found only one that yielded a greater than fivefold reduction in the target mRNA (Fig. 3a; Ref. 42). Thus, only 3% of the antisense molecules tested in this system were highly effective (Fig. 3b); 40% had almost no effect<sup>42</sup>.

Like those of ODNs, ribozyme target sites also vary in their accessibility. Chen and co-workers<sup>43</sup> directly demonstrated that cellular proteins and ribonucleoprotein complexes, such as ribosomes, can prevent ribozyme-mediated cleavage. They showed that a reporter gene was ribozyme-insensitive in wild-type *Escherichia coli* but was ribozyme-sensitive in a 'slow ribosome' mutant. In an accompanying editorial, John Burke (University of Vermont) remarked, 'The simple picture of ribozymes diffusing to, binding, and then cleaving an unstructured RNA is hopelessly oversimplistic'<sup>44</sup>.

#### Rational and Irrational design strategies are converging

At any one moment, a combination of the inherent structure of the RNA and its collection of bound proteins limits the number of accessible sites on RNA molecules, thereby providing a basis for specificity. Binding is the rare exception rather than the rule, and antisense molecules are excluded from most complementary sites (see Fig. 4). Since accessibility cannot be predicted, rational design of antisense molecules is not possible. Because design rules are lacking, effective antisense molecules are typically selected from 20-50 candidates

in a time-consuming and expensive process that promises to become even more elaborate. If tests of 50 molecules identify good candidates, tests of thousands of compounds should identify better ones. If thousands are to be tested, how should they be designed? Should their sequences be based solely on their potential to form a linear series of Watson-Crick base pairs with the target, or should nucleation sites be included, as they are in naturally occurring antisense RNAs (Ref. 45)? What about non-canonical base-pair interactions, and structural features such stem loops?

The relationship between accessibility to ODN binding *in vitro* and vulnerability to ODN-mediated antisense inhibition *in vivo* is beginning to be explored, and will continue to be an active area of research in the future. It is not yet clear whether *in vitro* screening techniques of the sort used by Milner and co-workers<sup>41</sup> will identify ODNs that are effective *in vivo*. With so many possible sequences to choose from, and the likelihood that *in vitro* studies will not always predict *in vivo* efficacy, straightforward new screening techniques need to be developed for use in cells.

#### Conclusions

The original concept that ODNs and ribozymes are exquisitely specific and easy to design has been jolted by the discovery of numerous mechanisms of action, leading to non-antisense effects, and the finding that most Watson-Crick binding sites in intended target RNAs

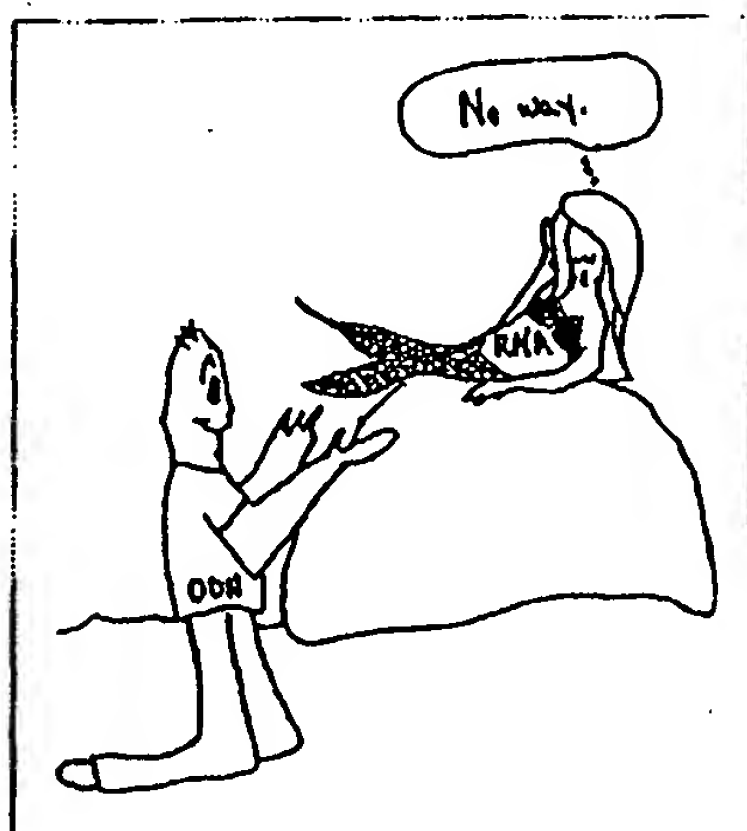


Figure 4

The structure of most potential target sites makes them inaccessible to anti-sense molecules and ribozymes.

are inaccessible. The time and expense necessary to screen large numbers of potential antisense molecules and ribozymes, and to carefully monitor their *in vivo* effects, raise the stakes for those seeking to use them as genetic probes. Although questions of their ultimate specificity remain, there is growing evidence that antisense molecules can be useful pharmacological tools when applied carefully<sup>17</sup>. In addition, certain non-antisense effects promise to be valuable therapeutically and will be fascinating to investigate. Because non-antisense effects are not currently predictable, rules for rational design cannot be applied to the production of non-antisense drugs. These effects must be explored on a case-by-case basis.

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# Suppression of Gene Expression by Targeted Disruption of Messenger RNA: Available Options and Current Strategies

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**Key Words.** RNA · DNA oligonucleotide · Hammerhead ribozymes · Hairpin ribozymes · Gene targeting · Targeted gene disruption · Antisense

## ABSTRACT

At least three different approaches may be used for gene targeting including: A) gene knockout by homologous recombination; B) employment of synthetic oligonucleotides capable of hybridizing with DNA or RNA, and C) use of polyamides and other natural DNA-bonding molecules called lexitropsins.

Targeting mRNA is attractive because mRNA is more accessible than the corresponding gene. Three basic strategies have emerged for this purpose, the most familiar being to introduce antisense nucleic acids into a cell in the hopes that they will form Watson-Crick base pairs with the targeted gene's mRNA. Duplexed mRNA cannot be translated, and almost certainly initiates processes which lead to its destruction. The antisense nucleic acid can take the form of RNA expressed from a vector which has been transfected into the cell, or take the form of a DNA or RNA oligonucleotide which can be introduced into cells through a variety of means. DNA and RNA oligonucleotides can be modified for stability as well as engineered to contain inherent cleaving activity.

It has also been hypothesized that because RNA and DNA are very similar chemical compounds, DNA molecules with enzymatic activity could also be developed. This assumption proved correct and led to the development of a "general-purpose" RNA-cleaving DNA enzyme. The attraction of DNazymes over ribozymes is that they are very inexpensive to make and that because they are composed of DNA and not RNA, they are inherently more stable than ribozymes.

Although mRNA targeting is impeccable in theory, many additional considerations must be taken into account in applying these strategies in living cells including mRNA site selection, drug delivery and intracellular localization of the antisense agent. Nevertheless, the ongoing revolution in cell and molecular biology, combined with advances in the emerging disciplines of genomics and informatics, has made the concept of nontoxic, cancer-specific therapies more viable than ever and continues to drive interest in this field. *Stem Cells* 2000;18:307-319

## INTRODUCTION

The notion that gene expression could be modified through use of exogenous nucleic acids derives from studies by *Paterson et al.* who first used single-stranded DNA to inhibit translation of a complementary RNA in a cell-free system in 1977 [1]. One year later, *Zamecnik* and *Stephenson* noted that a short (13nt) DNA oligonucleotide reverse complementary in sequence (antisense) to the Rous

sarcoma virus could inhibit viral replication in culture [2]. This observation is credited as being among the first to suggest the therapeutic utility of antisense nucleic acids, a concept which ultimately led to the awarding of a Lasker Prize in Medicine to *Dr. Zamecnik*. In the mid 1980s, the existence of naturally occurring antisense RNAs and their role in regulating gene expression was demonstrated [3-5]. These observations were particularly important because the

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fact that naturally occurring antisense nucleic acids played a role in regulating gene expression lent support to the belief that exogenously introduced reverse complementary nucleic acids might be utilized to manipulate gene expression in living cells. These seminal papers, and the literally thousands which have followed, have stimulated the development of technologies employing nucleic acids to manipulate gene expression. Virtually all available methods rely on some type of nucleotide sequence recognition for targeting specificity, but differ where and how they perturb the flow of genetic information [6]. Simply stated, strategies for modulating gene expression may be thought of as being targeted to the gene itself, or to the gene's messenger RNA (mRNA). Since this review will be focused on strategies aimed at disrupting the use of mRNA, antigene strategies will be addressed only briefly and mainly for the sake of completeness.

#### ANTIGENE STRATEGIES

At least three different approaches may be utilized for direct gene targeting. The "gold standard" is the gene "knock-out" achieved by homologous recombination [7, 8]. This approach results in the actual physical disruption of the targeted gene as a result of crossover events which occur during cell division between the targeting vector and the gene selected for destruction (Fig. 1A). Homologous recombination is extremely powerful, but the technique is hampered by the fact that it remains inherently inefficient, time-consuming, and expensive. While improvement in the efficiency of this process has been achieved [9, 10], this is a method which remains restricted to use in cell lines and animal models, if for no other reason than selection is required to find the cells in which the desired events have taken place. In clinical situations where high efficiency gene disruptions are required, it seems unlikely that this approach will serve as a useful therapeutic modality anytime in the foreseeable future.

A second option for gene targeting employs synthetic oligodeoxynucleotides (ODN) capable of hybridizing with double-stranded DNA [11-13]. Such hybrids are typically formed within the major groove of the helix, though hybridization within the minor groove has also been reported [14]. In either case, a triple-stranded molecule is produced, hence the origin of the term triple helix-forming oligodeoxynucleotide (TFO) (Fig. 1B). TFOs do not destroy a gene but prevent its transcription either by preventing unwinding of the duplex or preventing binding of transcription factors to the gene's promoter. TFO sequence requirements are based on the need for each base comprising the TFO to form two hydrogen bonds (Hoogsteen bonds) with its complementary base in the duplex. This

constrains TFOs to hybridization with the purine bases composing polypurine-polypyrimidine tracks within the DNA. The targeting efficiency of TFOs is further constrained by a number of factors, including need for divalent cations, and perhaps most importantly, by access to DNA compacted within the chromosome structure. Recent experiments from Wang *et al.* and Kochetkova *et al.* have provided evidence that triple helix formation can occur in living cells, suggesting that these difficulties may ultimately be overcome [15-17]. If shown practical, it has also been postulated that TFOs may prove useful in the treatment of certain genetic disorders such as sickle cell anemia, and hemophilia B, where their ability to trigger repair mechanisms might be used to correct single base pair mutations responsible for the disease [15, 18-20].

Final approaches worth mentioning are the use of specific nucleic acid sequences to act as "decoys" for transcription factors [21, 22], and the use of polyamides and

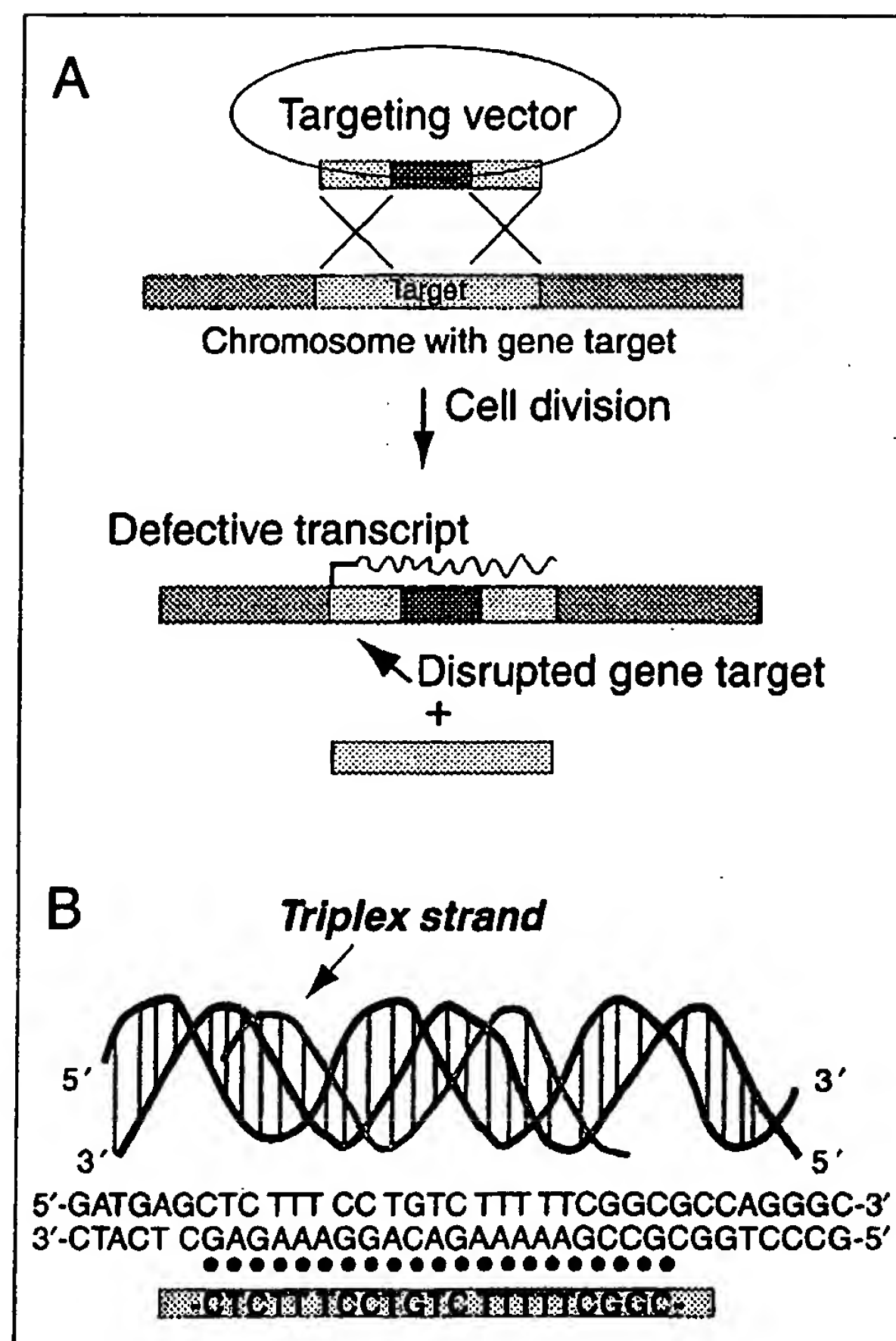


Figure 1. A) Targeting vector; B) Triplex strand. Adapted from [6].

other natural DNA-binding molecules called lexitropsins, that bind to specific bases in the minor groove of DNA [23, 24]. The use of decoy molecules evolves from the knowledge that transcription factor proteins recognize and bind specific DNA sequences. In theory then, it is possible to synthesize nucleic acids which will effectively compete with the native DNA sequences for available transcription factor proteins *in vivo*. If effective, the rate of transcription of the genes dependent on the particular factor involved will diminish. Unless single gene transcription factors can be identified, it is difficult to conceive how this approach, though potentially effective for controlling cell growth, can be made gene-specific. The polyamide approach may prove feasible since sequence-specific molecules can likely be designed and it appears that molecules of this type can easily access DNA within the chromosomes [23-25].

#### ANTI-MRNA STRATEGIES

A gene may be effectively "silenced" by destabilizing its mRNA, thereby preventing synthesis of the protein it encodes. Targeting mRNA, while less favorable than anti-gene strategies from a stoichiometric point of view, is nonetheless attractive because mRNA is in theory more accessible. Three basic strategies have emerged for this purpose. One employs an oligonucleotide that acts as an alternate binding site, or "decoy," for protein-stabilizing elements that normally interact with a given mRNA [26, 27]. By attracting away mRNA-stabilizing protein, the decoy induces instability, and ultimately destruction, of the mRNA. A newly developing approach is to affect RNA interference (RNAi) or post-transcriptional gene silencing [28, 29]. RNAi employs a gene-specific double-stranded RNA which, when introduced into a cell, leads to diminution of the targeted mRNA. The actual mechanism whereby this is accomplished is presently unknown but is under intense investigation with several clues being deciphered already [30, 31] including size and necessity for processing of the targeting dsRNA. In *C. elegans* and *Drosophila* this is a highly reproducible method for disrupting gene expression. Some reports suggest that this technique can be adapted for use in mammalian cells [32], but this remains uncertain at the moment. Finally, there is the more familiar, and more widely applied "antisense" strategy. We will focus on the latter.

Antisense (reverse complementary) nucleic acids are introduced into a cell in hopes that they will form Watson-Crick base pairs with the targeted gene's mRNA. As stated above, duplexed mRNA cannot be translated, and almost certainly initiates processes which lead to its destruction. The antisense nucleic acid can take the form of RNA expressed from a vector which has been transfected

into the cell [33], or take the form of a DNA or RNA oligonucleotide which can be introduced into cells through a variety of means. DNA and RNA oligonucleotides can be modified for stability as well as engineered to contain inherent cleaving activity [34, 35]. A number of these issues will be discussed in more detail in the sections below.

#### Antisense Oligonucleotides (AS-ONs)

AS-ONs are short stretches of nucleotides that are complementary to a region of targeted mRNA and can specifically suppress expression of that particular transcript. The following discussion will focus on the fundamental concepts concerning AS-ONs and their mechanisms of action. Examples of AS-ON use in experimental and clinical settings have been recently reviewed [36-38].

The exact mechanism of AS-ON action remains unclear, but it is known to be different for various types of AS-ONs. Generally, these molecules block gene expression by hybridizing to the target mRNA, resulting in subsequent double-helix formation. This process can occur at any point between the conclusion of transcription and initiation of translation, or even possibly during translation. Disruption of splicing, transport, or translation of the transcripts are all possible mechanisms, as is stability of transcript. Therefore, a major question is whether AS-ONs are most effective in the cytoplasm or nucleus. In the case of antisense oligodeoxyribonucleotides (AS-ODNs), cellular RNase H is able to bind to the DNA-RNA duplex and hydrolyze the RNA, resulting in increased transcript turnover. Any modification to the deoxy moiety at the 2'-sugar position prohibits RNase H action.

Modified AS-ONs or AS-ON analogs are often employed for *in vivo* antisense applications due to their increased stability and nuclease resistance. A longer serum half-life ensures that the AS-ON has ample time to reach and interact with its target mRNA. Phosphorothioate AS-ODNs are most widely used due to their long serum half-life and the fact that they are a suitable RNase H substrate. However, phosphorothioates display high affinity for various cellular proteins, which can result in sequence-nonspecific effects [39, 40]. Furthermore, high concentrations of phosphorothioates inhibit DNA polymerases and RNase H, which may render them ineffective as antisense agents [41]. Interestingly, many AS-ONs with 2'-modifications with groups such as O-methyl, fluoro, O-propyl, O-allyl, or many others exhibit greater duplex stability with their target mRNA along with antisense effects independent of RNase H (Fig. 1). These modifications create bulk at the 2' position, causing steric hindrance to play a significant role in increasing nuclease resistance. Nucleotide analogs

generally are also nuclease-resistant and often demonstrate superior hybridization properties due to modified backbone charge, although they usually are not acceptable substrates for RNase H. One example is peptide nucleic acid (PNA) where the sugar-phosphate moiety has been replaced by 2-aminoethyl glycine carbonyl units [42]. To these units are attached nucleotide bases spaced equally apart to DNA nucleotide bases. Instead of phosphodiester linkages between nucleotides, peptide bonds join the monomers to create a backbone neutral in charge. Not only do PNA oligomers hybridize to complementary DNA and RNA by Watson-Crick base pairing, they do so more quickly [43] and with greater affinity [42-44] because of the neutral backbone. In addition, PNAs are better at discriminating between base pair mismatches [44] and form less nonsequence-specific associations with proteins than phosphorothioate oligonucleotides [45]. Positive charges can also be introduced to backbone structure as in the case of (2-aminoethyl)phosphonates. Increased stability of duplex formation with both RNA and DNA has been reported with hybrid stability being more pH-dependent and less salt-dependent than natural RNA or DNA duplexes [46].

Some insight into the mechanism of AS-ON action has emerged recently through the work of Baker and colleagues (unpublished). Differences in ability to inhibit gene expression occur when either 2'-modified AS-ONs or 2'-unmodified AS-ONs are targeted to the exon 9 region of interleukin 5 (IL-5). Two forms of IL-5 exist: a soluble IL-5 lacking the exon 9 region, and a membrane-bound form, which contains exon 9. When unmodified AS-ONs are targeted to exon 9 of the IL-5 transcript, the expression of both membrane-bound and soluble IL-5 is inhibited. However, 2'-modified AS-ONs only suppress membrane-bound IL-5 expression. These observations seem to suggest that RNase H-dependent antisense effects are a nuclear event prior to splicing, whereas RNase H-independent oligonucleotides may affect splicing in transcript processing or may suppress gene expression after splicing has taken place. Additional evidence demonstrates that in the absence of RNase H activity, antisense effects may be a result of interference with translational initiation complex formation for certain types of 2'-modified AS-ON such as 2'-O-(2-methoxy)ethyl AS-ONs [47].

### Ribozymes

Naturally occurring ribozymes are catalytic RNA molecules that have the ability to cleave phosphodiester linkages without the aid of protein-based enzymes. This property has been exploited to specifically inhibit gene expression by targeting mRNA for catalytic cleavage especially in viral, cancer, and genetic disease therapeutics [48].

Similar to AS-ONs, ribozymes bind to substrate RNA through Watson-Crick base pairing, which offers sequence-specific cleavage of transcripts. Ideally, these agents should trigger enhanced transcript turnover as compared to RNase H-mediated AS-ON degradation of transcripts, considering ribozymes act through bimolecular kinetics (association of ribozyme and target transcript) whereas RNase H-dependent AS-ONs rely on trimolecular kinetics (association of AS-ON, target transcript, and RNase H). Since ribozymes are RNase H-independent, 2'-modifications to increase stability do not diminish antisense effects and experiments have shown some modifications do not attenuate catalytic ability [49]. Unlike AS-ONs, ribozymes can be expressed from a vector, which offers the advantage of continued production of these molecules intracellularly [50, 51]. However, stable transformation of cells *in vivo* has its own complications and will not be discussed in this review.

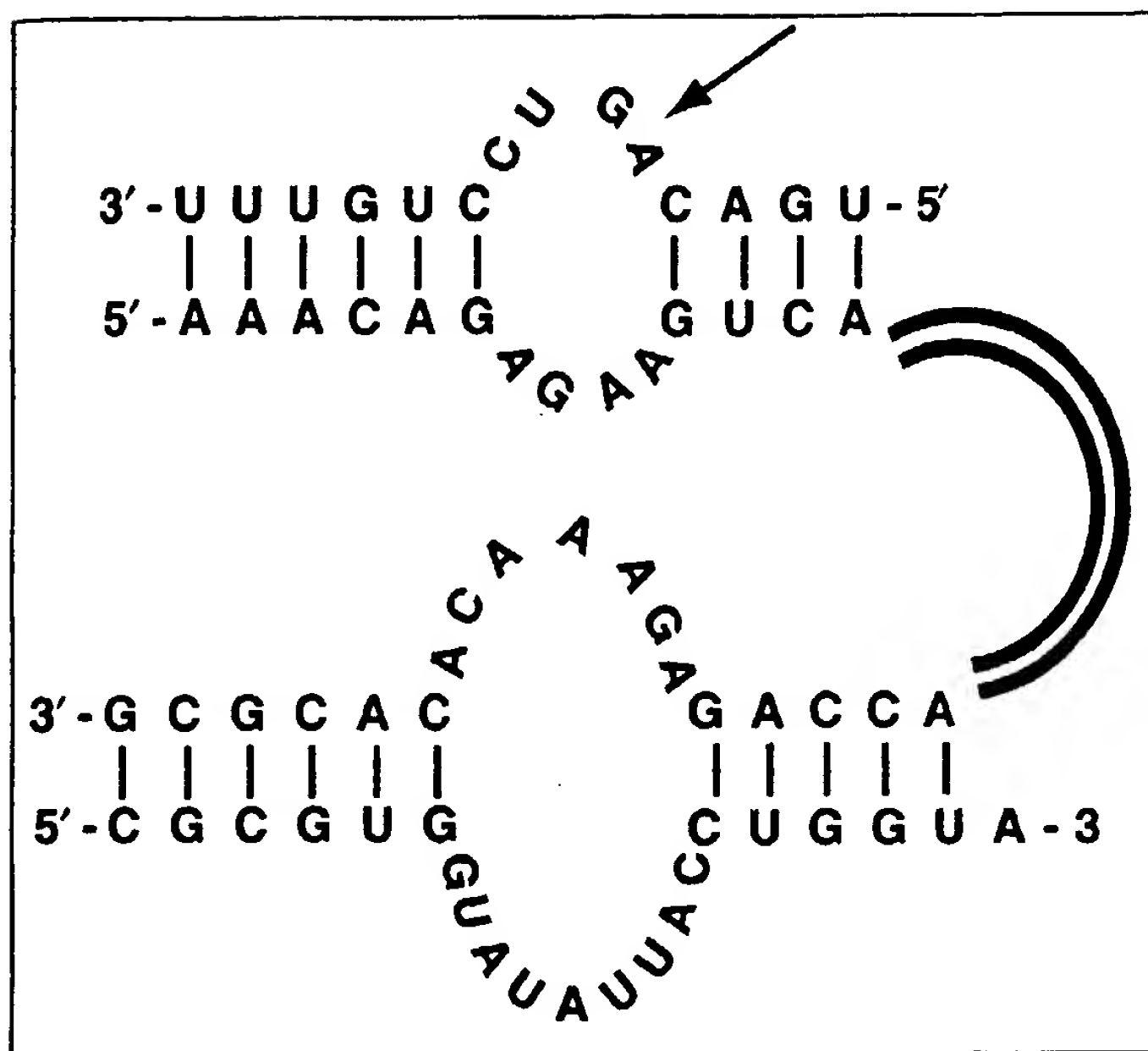
If ribozymes are to perform effectively as "enzymes," they must not only bind substrate RNA but also dissociate from the cleavage product in order to act on additional substrates. Studies suggest that in some cases, dissociation of cleavage product may be the rate-limiting step [52, 53]. Furthermore, some ribozymes require high divalent metal ion concentrations for efficient substrate cleavage, which may limit their use in intracellular environments [54]. All of these concerns need to be addressed and overcome in order for ribozymes to have a future in medical therapy. Two ribozymes, the hammerhead ribozyme and the hairpin ribozyme, have been extensively studied due to their small size and rapid kinetics. Their application has been recently reviewed in several publications [55-59].

### Hammerhead Ribozymes

The hammerhead ribozyme consists of a highly conserved catalytic core, which will cleave substrate RNA at NUH triplets 3' to the H, where N is any nucleotide, U is uracil, and H is any nucleotide but guanine (Fig. 2) [34]. In fact RNA cleavage may be less restricted since recent studies demonstrate exceptions to the "NUH" rule. Investigators have established that cleavage can actually occur 3' to any NHH triplet [59]. Furthermore, *in vitro* selection protocols have made it possible to screen for ribozymes with various cleavage specificities including one that cleaves at AUG sites [60]. Thus, the limitations for sequence specificity of triplet-cleavage sites on the target RNA are less than previously thought. In addition to the catalytic core, a particular cleavage site in a target RNA can be specifically recognized by the hammerhead ribozyme arms. By creating complementary sequences in the arms to sequences flanking the cleavage site, the ribozyme will hybridize specifically to the RNA of interest.







**Figure 3. Hairpin ribozyme in the docked position.** The two loop regions associate with each other in order to cleave the substrate RNA. Arrow indicates position of cleavage. Adapted from [58].

DNA molecules with enzymatic activity could also be developed [76]. This assumption proved correct and led to the development of a "general-purpose" RNA-cleaving DNA enzyme [77]. The molecule was identified from a library of >1,000 different DNA molecules by successive rounds of in vitro selective amplification based on the ability of individual molecules to promote  $Mg^{2+}$ -dependent, multiturnover, cleavage of an RNA target.

The selected molecule was named the "10-23 DNA enzyme," because it was derived from the 23rd clone obtained after the 10th round of selec-

inhibit hairpin ribozyme cleavage depending on metal ion conditions [74]. In the presence of magnesium, aminoglycoside antibiotics inhibit ribozyme cleavage with the degree of inhibition depending on the binding affinity of the antibiotic to the ribozyme. However, in the absence of metal ions, aminoglycoside antibiotics prove to assist cleavage with an optimum reaction condition at pH 5.5 and poorer kinetics as the pH is increased, exactly opposite to trends observed for magnesium. In this case, the metal ions are most likely being replaced by the amino groups of these antibiotics.

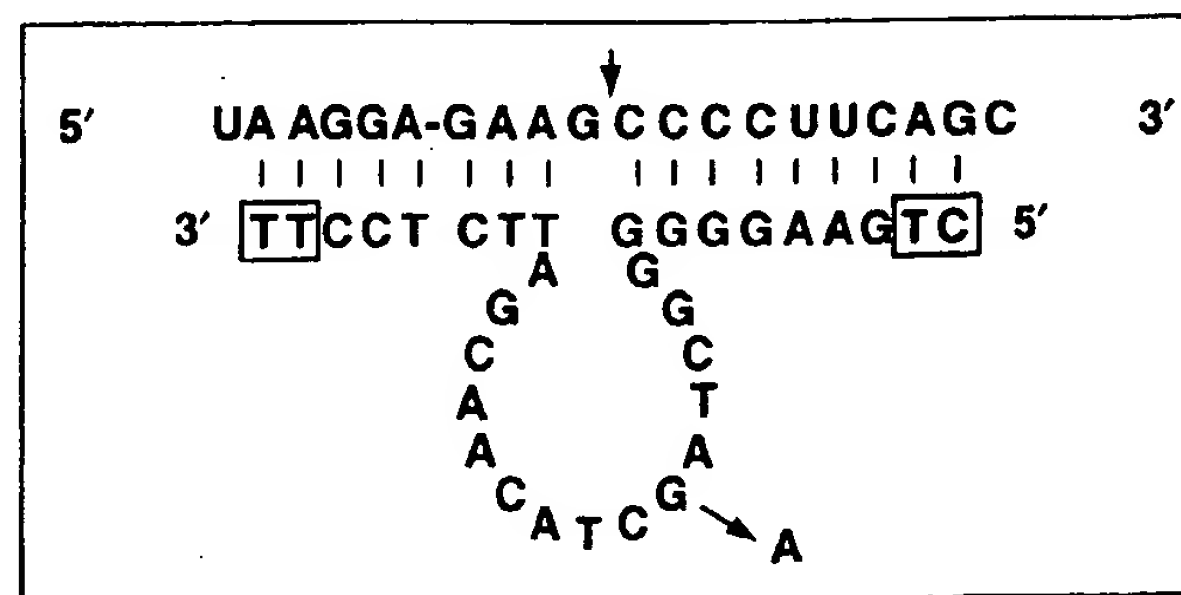
Polyamines such as spermidine and spermine have also been reported to support hairpin ribozyme cleavage ability. In the absence of magnesium, spermidine allows the cleavage reaction to persist at very slow kinetics compared to magnesium alone [72]. However, spermine alone gives very efficient cleavage of RNA comparable to that of magnesium, and when in the presence of low magnesium concentrations similar to intracellular conditions, spermine displays considerable increase in cleavage rates [74]. The fact that spermine is the major polyamine in eukaryotic cells may explain why the hairpin ribozyme has shown remarkable intracellular cleavage activity in mammalian cells and may make future therapeutic endeavors with the hairpin ribozyme much easier [75].

#### DNAzymes

While investigating ways to improve the function of ribozymes, Breaker and Joyce made the assumption that because RNA and DNA are very similar chemical compounds,

selective amplification [77]. The "10-23 DNA enzyme" is composed of a catalytic domain of 15 deoxynucleotides, flanked by 2 substrate-recognition domains of ~8 nucleotides each (Fig. 4). The recognition domains provide the sequence information required for specific binding to an RNA substrate. They also supply the binding energy required to hold the RNA substrate within the active site of the enzyme. It is straightforward that by appropriately designing the flanking sequences, the DNAzyme can be made to cleave virtually any RNA that contains a purine-pyrimidine junction.

The attraction of DNAzymes over ribozymes is that they are very inexpensive to make and that because they are composed of DNA and not RNA, they are inherently more stable



**Figure 4. Complex formed by an mRNA (top strand) and a "10-23" DNAzyme (bottom strand).** Vertical arrow indicates the mRNA cleavage site. Replacement of G by A within the catalytic core of the DNAzyme (diagonal arrow) will eliminate its catalytic activity. Adapted from [77].

than ribozymes. Nevertheless, DNAzymes must ultimately overcome the same problems faced by ribozymes and oligonucleotides if they are to be effective in cellular systems (see below). These are stability, ability to be targeted to the cell of interest, ability to achieve sufficient intracellular concentration to cleave to the targeted mRNA, ability to hybridize with their mRNA target, and lack of toxicity. In this regard, many of the chemical modifications employed to stabilize ODNs can be incorporated into the 10-23 DNA enzyme without loss of activity. There is a suggestion from recent reports that issues of intracellular concentration and target hybridization may also be solvable [78, 79].

#### APPLICATION OF THE "ANTISENSE" STRATEGY

Although antisense interference methods appear impeccable in theory, many additional considerations must be taken into account in applying the strategy in living cells. Since both AS-ONs and ribozymes are considered oligonucleotides, quite often similar solutions can be offered to address the problems encountered. As mentioned earlier, increasing stability of antisense agents can be easily achieved through nucleotide modifications or analogs. However, additional considerations crucial to reliable experimental outcome include mRNA site selection, drug delivery, and intracellular localization of the antisense agent.

#### mRNA Site Selection

Within living cells, transcripts exist in low energy conformations in which secondary structures dominate in folding the linear polymer. In addition, interactions with cytoplasmic proteins produce further structural properties. The end result is that much of the mRNA sequence is hidden and only partial sequences within the total mRNA length are accessible for hybridization. RNA folding programs that generate three-dimensional folding patterns based on free energy calculations often give an unreliable depiction for in vivo relevance. Therefore, a good empirical method to probe for suitable sites is necessary.

A system to probe for suitable sites in mRNA for AS-ON or ribozyme-targeting has recently been established using RNase H cleavage as an indicator for accessibility of sequences within transcripts [80]. A mixture of ODNs that are complementary to certain regions of a transcript is added to cell extracts and exposed to RNase H. RT-PCR of the transcript can then be used to show which ODNs actually had access to the transcript and hybridized in order to create an RNase H-vulnerable site. Combining this methodology with computer-assisted sequence selection may enhance this approach as well [81].

Another technique currently being tested is the use of molecular beacons for site selection (Gewirtz *et al.*, unpublished). These molecules are ODNs with the ability to form stem-loops where the loops are targeted to regions of the transcript [82]. The stems have a fluorophore linked to either the 5' or 3' end and a quencher molecule is attached to the other so that in the stem-loop configuration, fluorescence is not observed due to the proximity of the quencher molecule to the fluorophore. However, when hybridization proceeds, the act of forming a double helix between the loop and the transcript causes unfolding of the stem-loop and brings the quencher and fluorophore apart in space. Thus, fluorescence should increase as a result of hybridization. Currently, these molecules are being applied to probe for accessible sites within mRNA with very encouraging results (Jen and Gewirtz, unpublished).

#### Delivery

One of the major limitations for the therapeutic use of AS-ONs and ribozymes is the problem of delivery. Import of these compounds into cells can be accomplished by exogenous delivery in which presynthesized oligonucleotides come in direct contact with the plasma membrane, resulting in subsequent cellular uptake [83]. Naked oligonucleotides are poorly incorporated into cells in this fashion and often require a vehicle for efficient delivery. In tissue culture, many classes of compounds have been used as delivery vehicles including cationic liposomes, cationic porphyrins, fusogenic peptides, and artificial virosomes. These compounds share the characteristic of forming complexes with oligonucleotides through electrostatic interactions between the negatively charged oligonucleotide phosphate groups and positive charges contained by the vehicles themselves. In addition, some degree of protection from nuclease degradation is conferred to the oligonucleotide when associated with such delivery vehicles. Other strategies including cell permeabilization with streptolysin-O and electroporation have been used [84] but are restricted in utility for clinical settings. Presently, some success has been achieved in tissue culture, but efficient delivery for in vivo animal studies remains questionable.

Cationic lipids form stable complexes with oligonucleotides, which exhibit improved cellular uptake [85-87]. The result is enhanced antisense activity. Further studies indicate that phosphorothioated ODNs dissociate from cationic lipids before entering the nucleus where it is free to hinder target transcript function [88]. These compounds have proven to be quite effective in cell culture and have been commercialized, but their relatively high cytotoxic properties may restrict their use.



Alternatives to cationic lipids are being explored. Recently, cationic porphyrins have proven to be effective vehicles for AS-ONs in tissue culture [89, 90]. Two cationic porphyrins used by Benimetskaya and colleagues, tetra(4-methylpyridyl) porphyrin (TMP) and tetraanilinium porphyrin (TAP), demonstrate properties important for AS-ON delivery. 5'-fluorescein-labeled phosphorothioates show that both TMP and TAP more efficiently deliver AS-ONs into cells than naked AS-ONs. Nuclear fluorescence is observed after porphyrin/AS-ON complex exposure to cells while fluorescein labeled AS-ONs alone are taken up into vesicular structures. Thus, cationic porphyrins not only help AS-ON delivery into the cell, but they are also able to localize the AS-ON in the nucleus where mRNA and RNase H are present. FRET studies on the ability of cationic porphyrins to quench 5'-fluorescein-labeled phosphorothioates suggest intracellular dissociation of the oligonucleotide from the porphyrin.

Fusogenic peptides form peptide cages around oligonucleotides in order to boost oligonucleotide uptake. Many of these peptides contain polylysine residues, which cause membrane destabilization [91]. Others are derived from viral proteins such as the fusion sequence of HIV gp41 [92] and hemagglutinin envelop protein [93, 94]. Generally, these agents are less cytotoxic than lipids but are still able to achieve similar delivery efficacy. Artificial virosomes are another class of delivery vehicles which take advantage of the natural ability of a virus to gain entry into cells. Reconstituted influenza virus envelopes known as virosomes can fuse with endosomal membranes after internalization through receptor-mediated endocytosis [95]. Recently, cationic lipids have been incorporated into virosome membranes to further aid delivery [96, 97].

Finally, Dheur and colleagues have noted that while oligonucleotides delivered with lipofectins usually do not elicit antisense activity (likely because cationic lipid formulations do not protect unmodified oligonucleotides from nuclease degradation), a cationic polymer, polyethylenimine (PEI) [98], improves the uptake and antisense activity of antisense phosphodiester oligodeoxynucleotides (PO-ODN) [99]. Interestingly, PEI-phosphorothioate (PS) ODN particles were efficiently taken up by cells but PS-ODN did not dissociate from the carrier. These investigators suggested that the low cost of PEI compared with cytofectins, the increased affinity for target mRNA and decreased affinity for proteins of PO-ODN compared with PS-ODN might make the use of PEI-PO-ODN very attractive.

#### Localization

In order for AS-ONs or ribozymes to suppress gene expression, they must be colocalized to the same intracellular

compartment as their target mRNA. Intracellular trafficking seems to play an important role in the fate of these molecules since their spatial distribution does not correspond to simple diffusion. Many factors determine localization patterns of AS-ON and ribozymes including the antisense agent itself, delivery vehicle, and targeted cell type. In addition, evidence for cell cycle-dependent localization patterns has been reported with nuclear localization predominantly in the G<sub>2</sub>/M phase [100].

mRNAs can exist in several cellular compartments including the cytoplasm, nucleus, and nucleolus. It remains unclear as to where oligonucleotides should be directed for most efficient antisense activity to occur, although endosomal localization usually predicts ineffective antisense response. The optimal site for mRNA degradation may be dependent on the type of antisense agent used [47]. Recently, ribozymes attached to small nucleolar RNAs (snoRNAs) called snoribozymes exhibited nearly 100% efficiency in cleaving a target RNA also localized to the nucleolus by snoRNA attachment [101]. Even though this particular experiment is based on cleavage of an artificial substrate, the expanding roles associated with the nucleolus may prove the nucleolus to be an important site to target mRNA degradation [102]. In another study, antisense RNA inserted within a variable region of ribosomal RNA (rRNA) proved to heighten ribozyme efficiency and may be due to colocalization of rRNA with mRNA [103].

#### ANTISENSE DRUG DESIGN

Certain issues to be aware of concerning antisense experimental design are quite important to the consistent and efficacious outcome of inhibiting gene expression. Even when the above considerations regarding the potential problems of antisense experiments are addressed, other factors may come into play especially involving antisense drug design. Only two will be mentioned here: formation of G quartets and chirality of modified oligonucleotides. Purine-rich oligonucleotides, especially ones containing four consecutive guanine residues, have a tendency to form stable tetrameric structures under physiologic conditions [104]. The guanosines of single-stranded oligonucleotides are not restrained in space by rigid double-helix structure and can therefore form various hydrogen bonds not observed in Watson-Crick base pairing. Tetraplexes known as G quartets arise as a result. Dissociation rates of these structures may be quite slow and may prevent hybridization of AS-ONs or ribozymes to their target transcript, rendering them ineffective as antisense agents. However, the absence of G quartet structures at 37°C under cellular salt conditions could mean that G quartet formation is irrelevant at physiologic temperatures [105].

Interestingly, nonsequence-specific gene inhibition by phosphorothioate oligonucleotides containing tetraquinosine tracts prove aptameric properties can play an important role in gene inhibition for some sequences of ONs [106].

Another important aspect to consider is the issue of chirality for certain oligonucleotides. Unmodified phosphodiester oligonucleotides do not have a chiral center at the phosphorous position. However, when a terminal oxygen of the phosphate is replaced by a sulfur, as in PS-ONs, the phosphorous gains chirality. The digestion kinetics of PS-ONs by 3'-exonucleases display bi-exponential decay with a fast and slow phase of digestion. These phases are due to stereoselectivity of the 3'-exonucleases on the chiral phosphorothioate center [107]. A 25-mer containing a 3'-terminal internucleotide linkage in the S-configuration degrades 300-fold slower than the same 25-mer with an R-configuration phosphorothioate linkage.

### CONCLUSIONS

The ongoing revolution in cell and molecular biology, combined with advances in the emerging disciplines of genomics and informatics, has made the concept of non-toxic, cancer-specific therapies more viable than ever. The recent development of a relatively specific biochemical inhibitor of the bcr/abl protein tyrosine kinase in patients with chronic myelogenous leukemia is a stunning example

of this principle [108]. For therapies focused on direct replacement, repair, or disabling of disease-causing genes, progress has been much slower and a successful equivalent to the biochemical bcr/abl inhibitor has yet to be achieved. In the case of anti-mRNA strategies, it is hoped that the above discussion will have made the reasons for this clearer. Given the state of the art, it is perhaps not surprising that effective and efficient clinical translation of the antisense strategy has proven elusive. While a number of phase I/II trials employing ONs have been reported [109-116], virtually all have been characterized by a lack of toxicity but only modest clinical effects. A recent paper by *Waters et al.* describing the use of a bcl-2-targeted ON in patients with non-Hodgkin's lymphoma is typical in this regard [117, 118].

The key challenges to this field have been outlined above. It is clear that they will have to be solved if this approach to specific antitumor therapy is to become a useful treatment approach. A large number of diverse and talented groups are working on this problem, and we can all hope that their efforts will help lead to establishment of this promising form of therapy.

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